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Mason et al.

(10) **Patent No.: US 8,252,574 B2**
(45) **Date of Patent: Aug. 28, 2012**

(54) **PSEUDOINFECTIOUS *FLAVIVIRUS* AND USES THEREOF**

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(73) Assignee: **The Board of Regents of the University of Texas System**, Austin, TX (US)

(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 512 days.

(21) Appl. No.: **11/711,532**

(22) Filed: **Feb. 27, 2007**

(65) **Prior Publication Data**

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Related U.S. Application Data

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(51) **Int. Cl.**
C12N 7/00 (2006.01)
A61K 39/12 (2006.01)

(52) **U.S. Cl.** **435/235.1**; 424/218.1; 424/205.1

(58) **Field of Classification Search** ..... None
See application file for complete search history.

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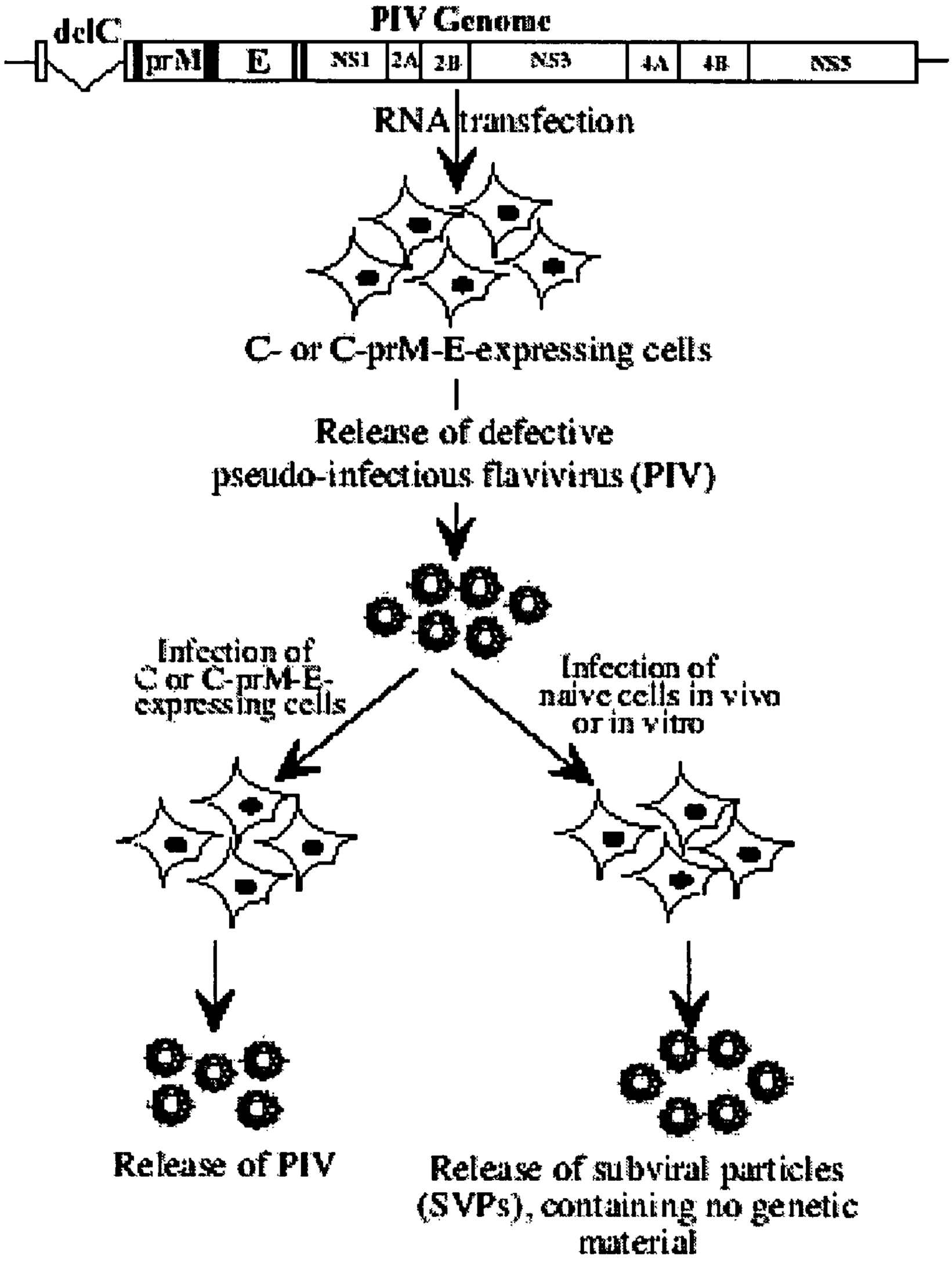
Primary Examiner — Stacy B. Chen

(57) **ABSTRACT**

The present invention discloses a replication-deficient pseudoinfective virus belonging to the Flaviviridae family that lack the capsid gene, where the replication-deficient pseudoinfective virus propagates only in cells expressing the capsid or capsid, prM and envelope protein of the flavivirus. The present also discloses the method of producing such viruses on a large scale and the use of these pseudoinfective viruses as vaccines for preventing diseases caused by infections of humans or animals by the viruses belonging to this family.

25 Claims, 29 Drawing Sheets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delC
prM
E
NS1
2A
2B
NS3
4A
4B
NS5
RNA transfection
C- or C-prM-E-expressing cells
Release of defective
pseudo-infectious flavivirus (PIV)
Infection of
C or C-prM-E-
expressing cells
Infection of
naive cells in vivo
or in vitro
Release of PIV
Release of subviral particles
(SVPs), containing no genetic
material

Fig.1

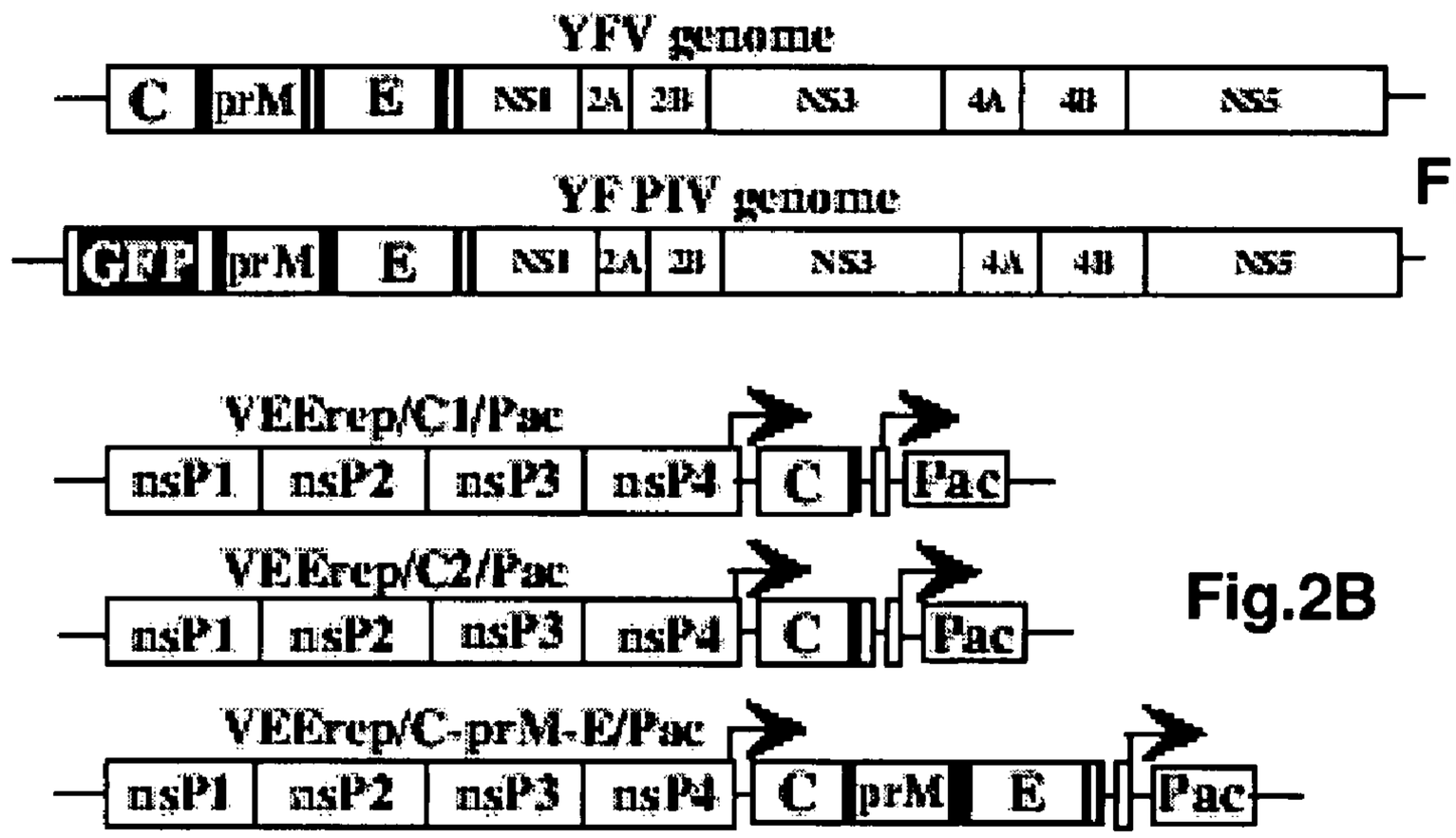


Fig.2A

Fig.2B

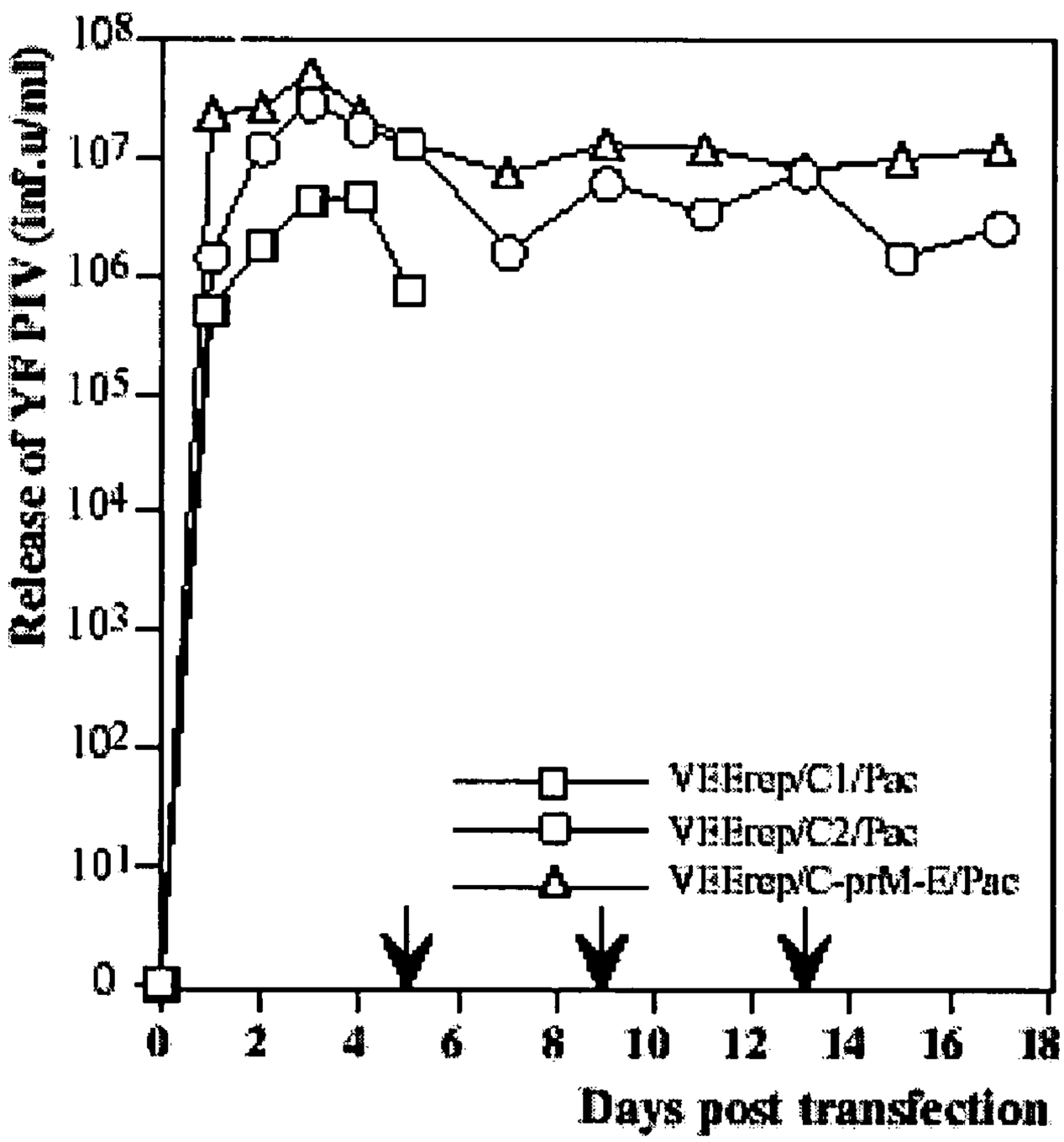
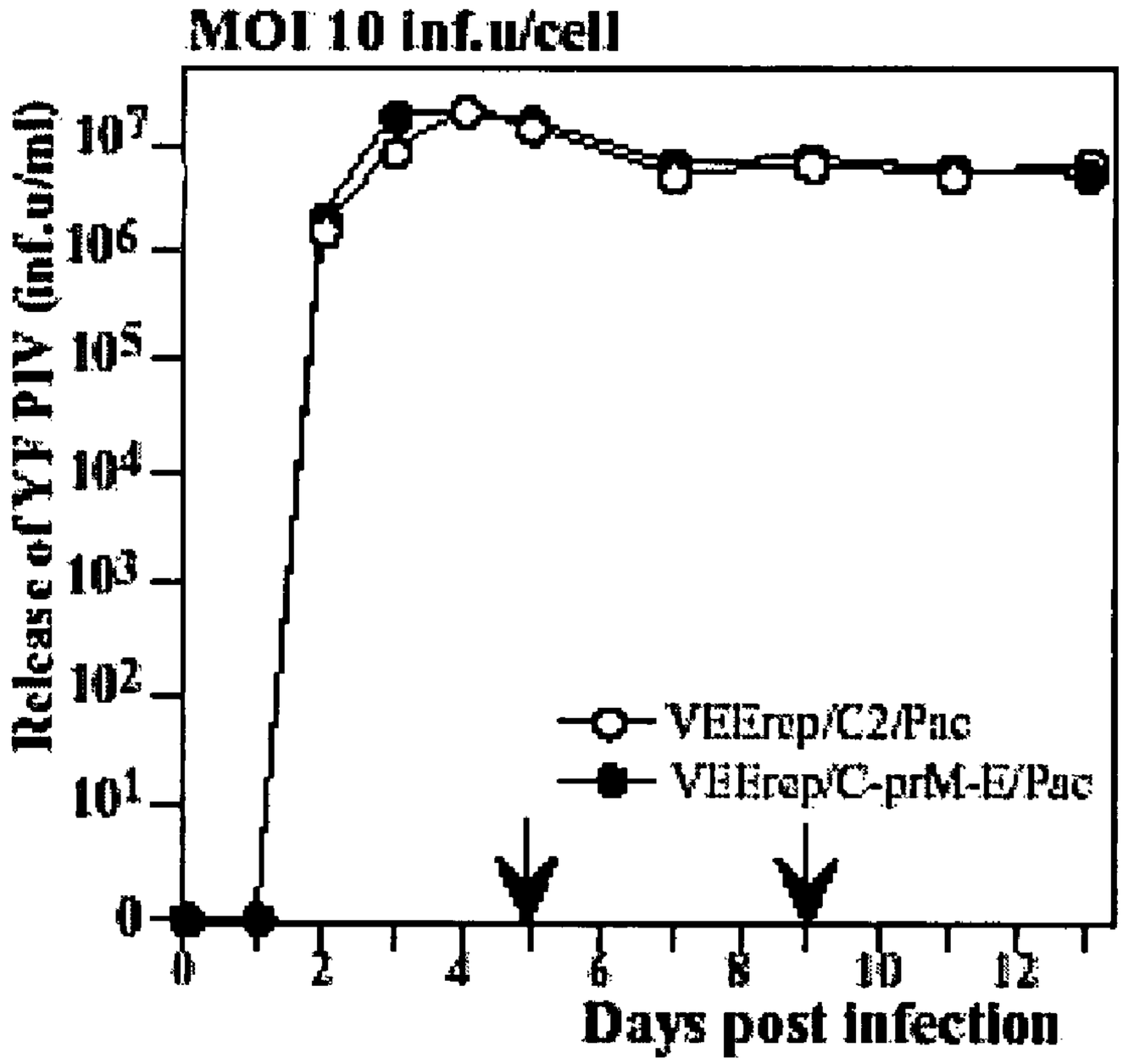


Fig.2C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Release of VEE PIV (inf.u/ml)
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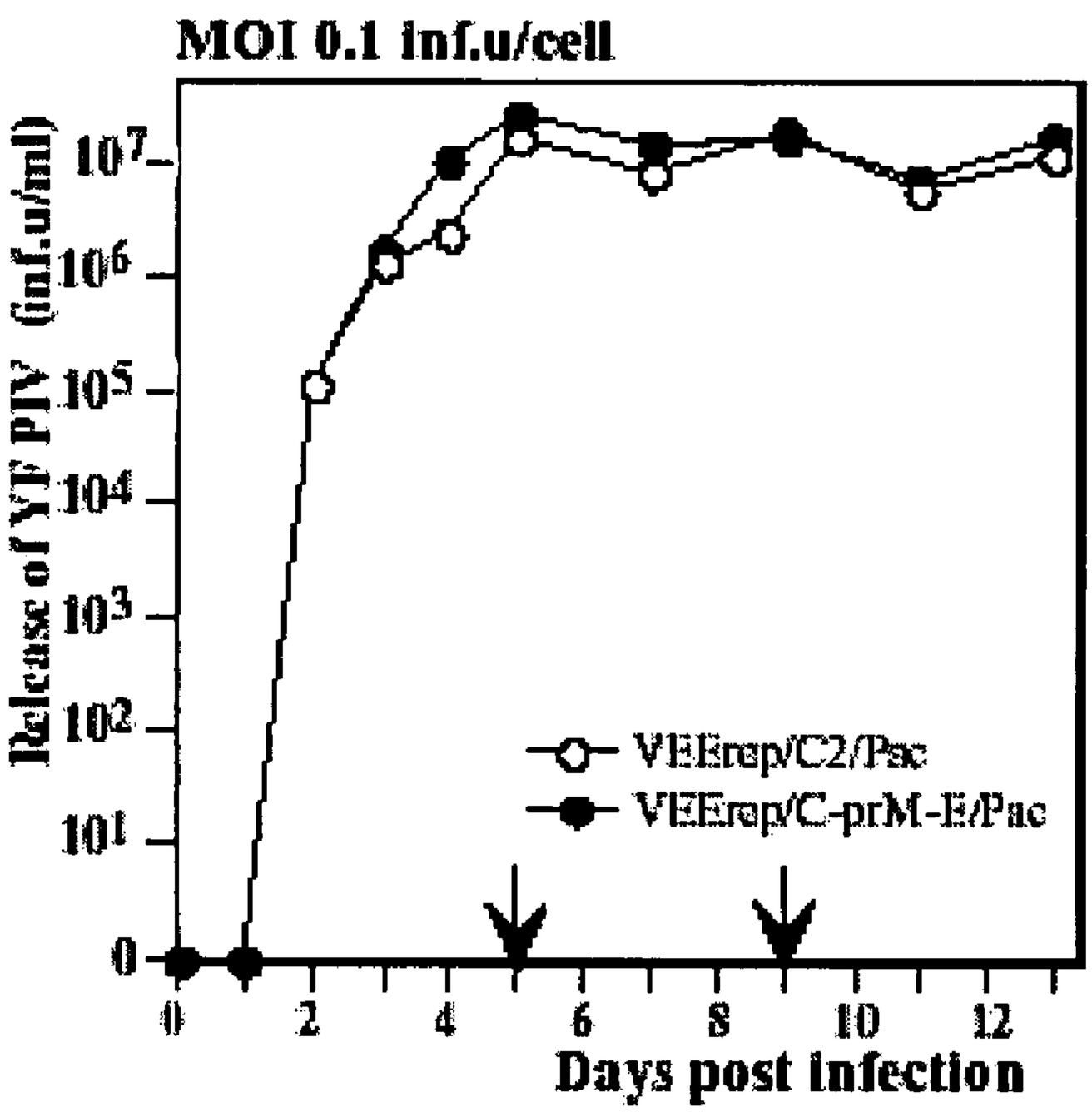
10^6
10^5
10^4
10^3
10^2
10^1
0
0
2
4
6
8
10
12
Days post infection
VEErep/C2/Pac
VEErep/C-prM-E/Pac

Fig. 3A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Release of VEE PIV (inf.u/ml)
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10^1
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0
2
4
6
8
10
12
Days post infection
VEErep/C2/Pac
VEErep/C-prM-E/Pac

Fig. 3B

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Fig. 4A

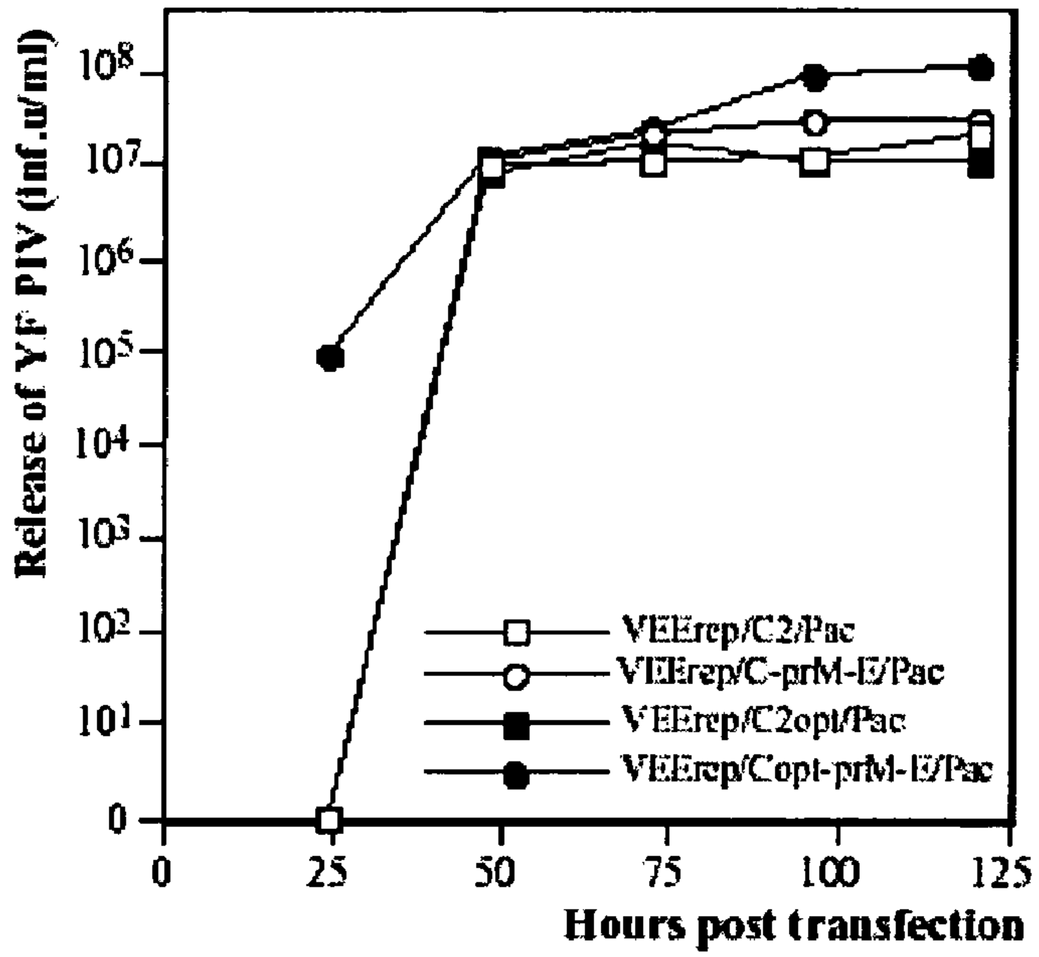


Fig. 4B

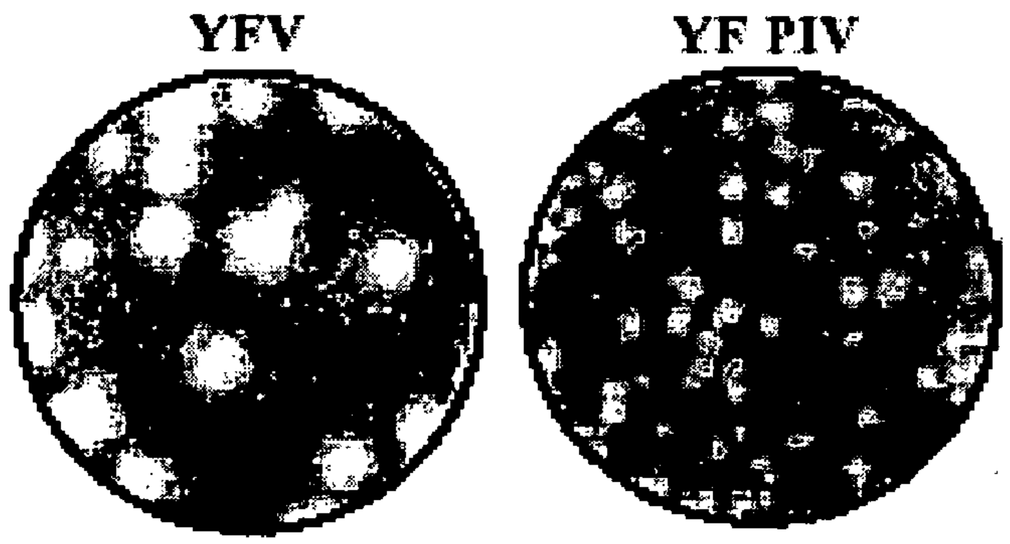
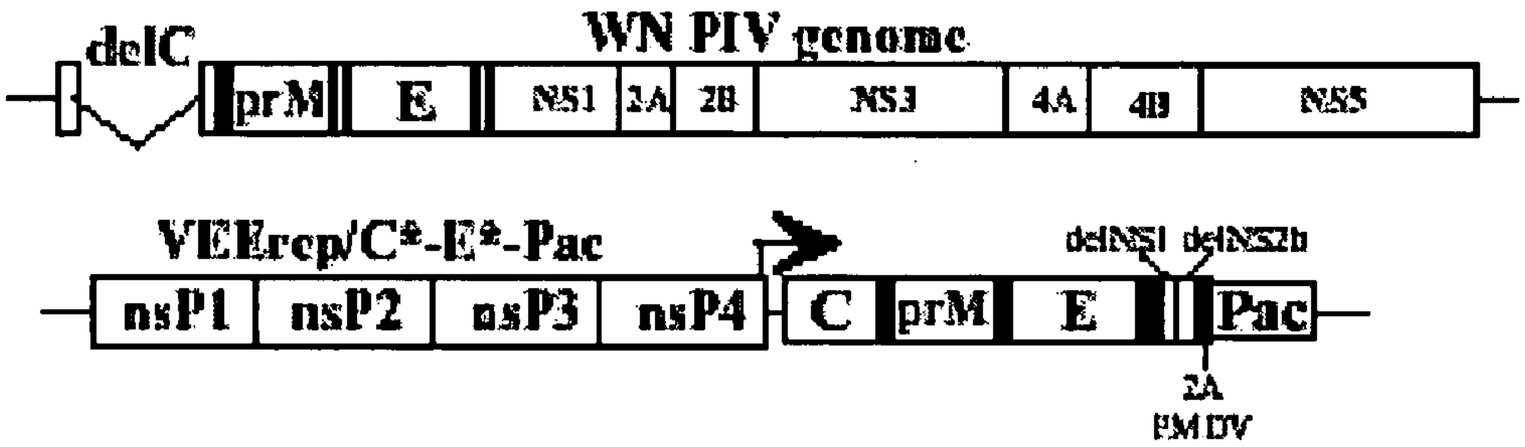


Fig. 4C

Fig. 5A



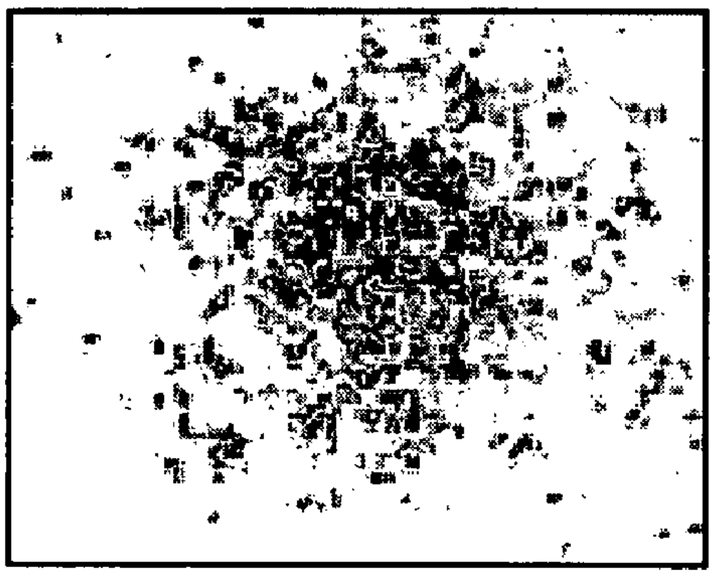


Fig. 5B

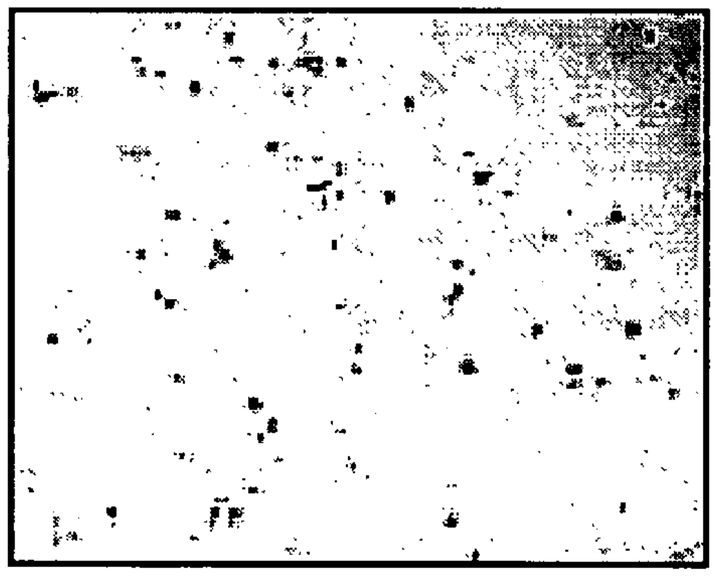


Fig. 5C

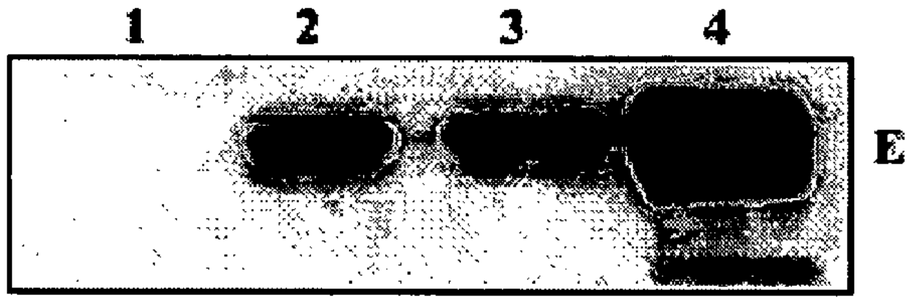


Fig. 6A

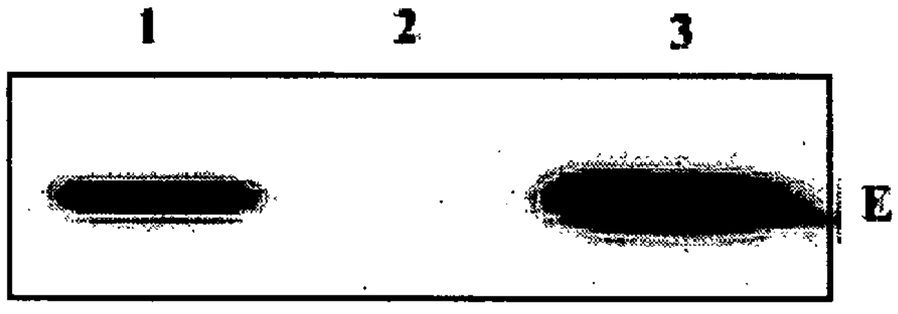
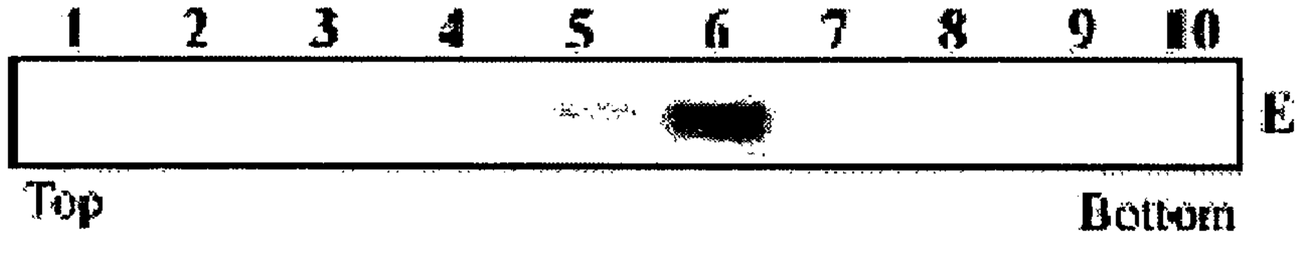


Fig. 6B

Fig. 6C



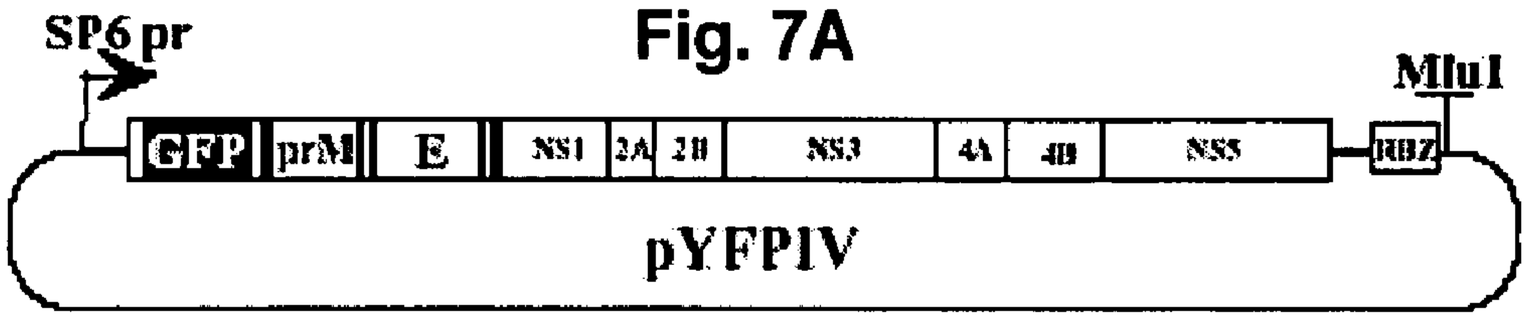


Fig. 7A

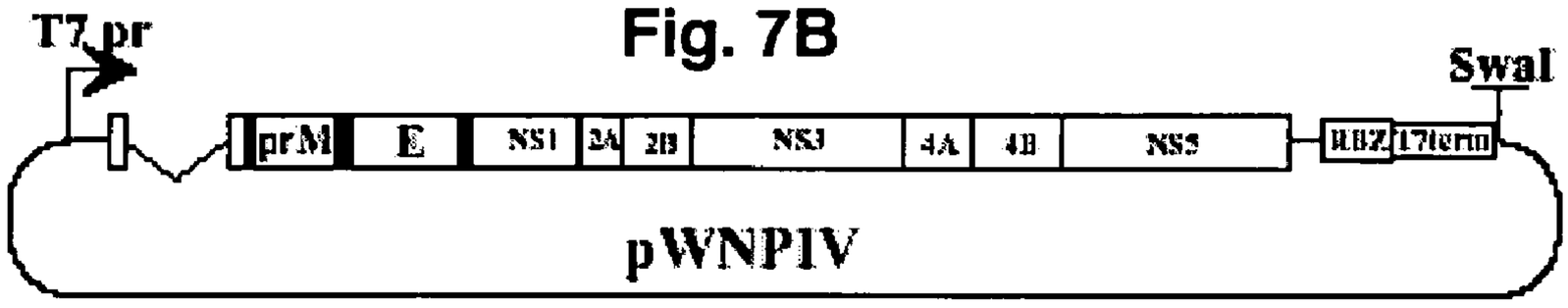


Fig. 7B

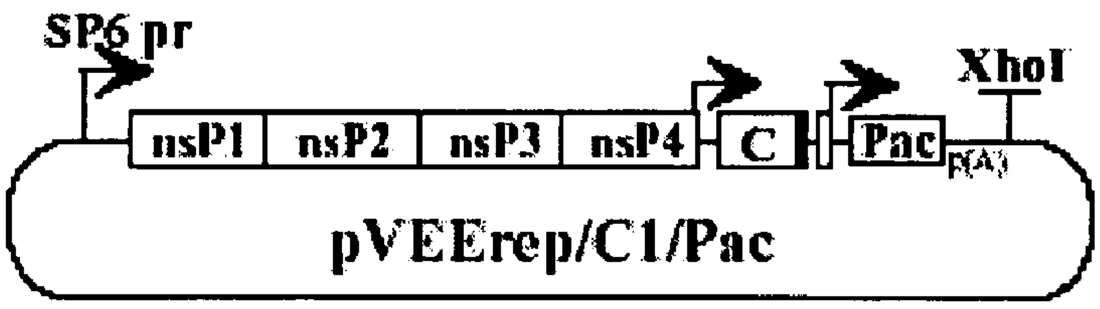


Fig. 7C

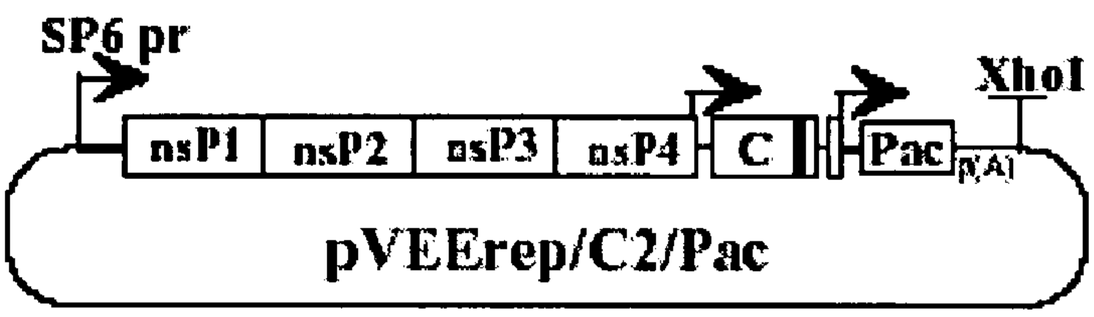
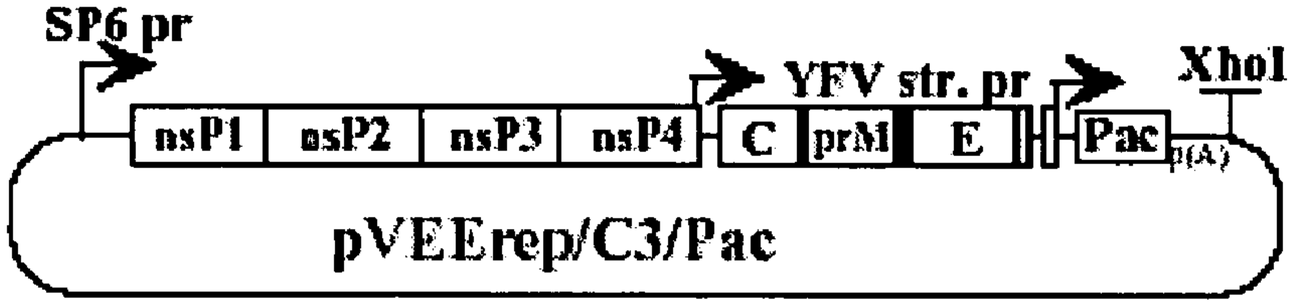


Fig. 7D



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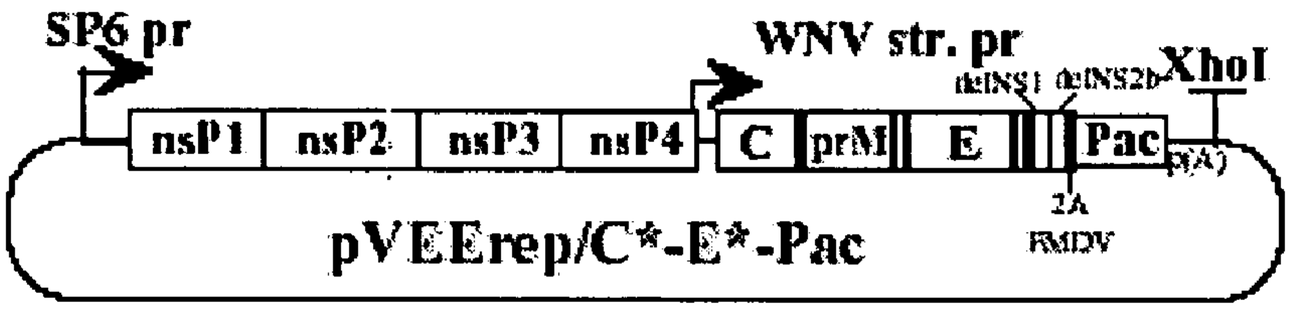

Fig. 7F

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Fig. 8A

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Fig.8B

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Fig. 8C

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Fig.8D

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Fig. 8E

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Fig. 8F

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Fig. 8G

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Fig. 8H

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Fig. 8I

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Fig. 8J

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Fig.8K

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Fig. 8L

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Fig. 8M

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Fig. 8N

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Fig. 8O

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Fig. 8P

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Fig. 8Q

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Fig. 8R

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Fig. 8S

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Fig. 8T

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gtttggctctgcctttcaggggctatttggcggcttgaactggataacaaaggtcatcatgggggcggtacttatatgggttggc
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gctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaaggtaactggcttcagcagagcgcagatac
caaatactgtccttctagtgtagccgtagttaggccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaa

Fig. 8U

tcctgttaccagtggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccggataaggcg
cagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaacgacctacaccgaactgagatacctа
cagcgtgagcattgagaaagcgccacgcttcccgaagggagaaaggcggacaggtatccggtaagcggcagggtcg
gaacaggagagcgcacgagggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctgac
ttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagcaacgcgagctcgatttaggtga
cactata **(SEQ ID NO: 11)**

Fig. 8V

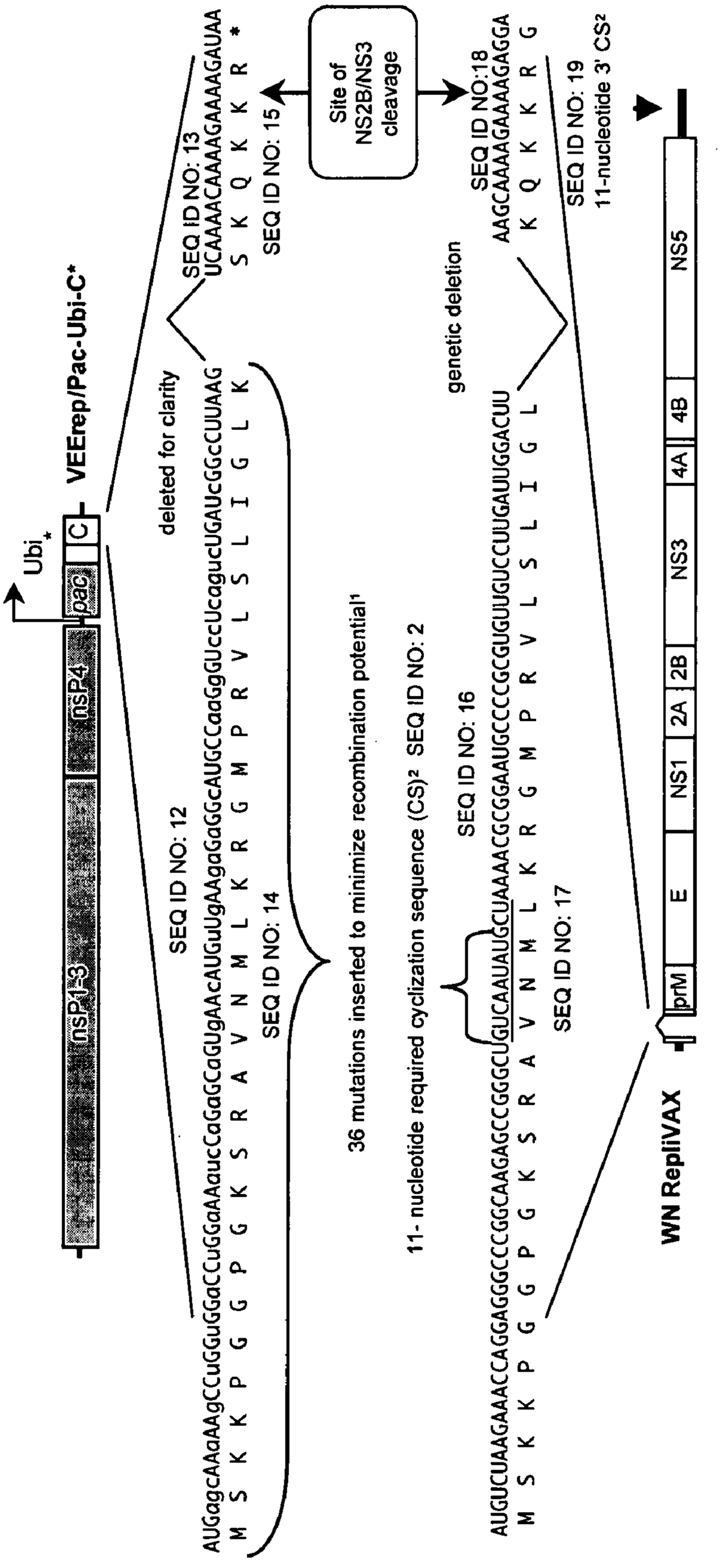
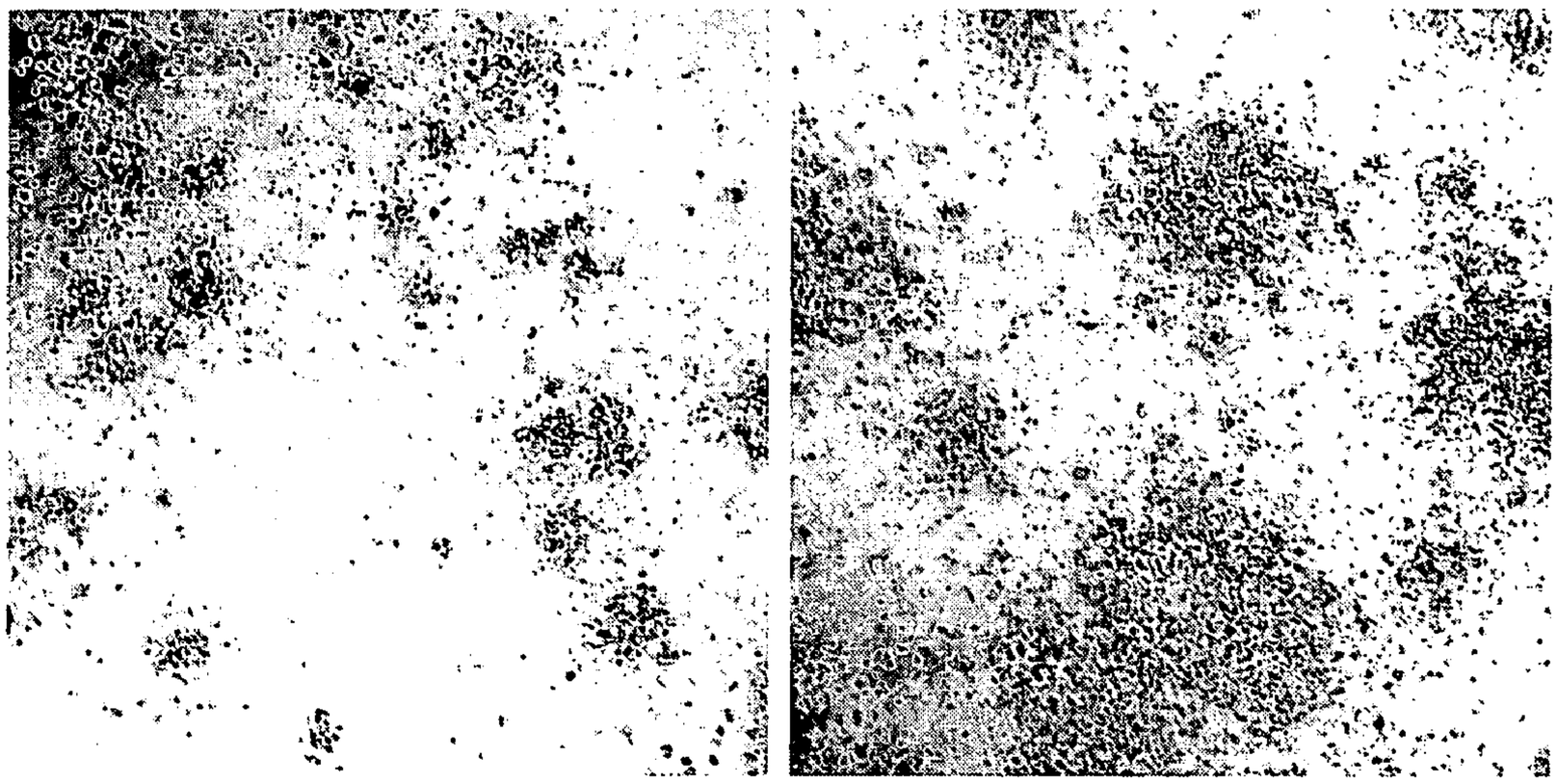

WN RepliVAX
prM
E
NS1
2A
2B
NS3
4A
4B
VEErep/Pac-Ubi-C*
Ubi*
pac
C
nsP1-3
nsP4
NS5
Site of NS2B/NS3 cleavage
SEQ ID NO: 12
SEQ ID NO: 14
SEQ ID NO: 15
SEQ ID NO: 16
SEQ ID NO: 17
SEQ ID NO: 18
SEQ ID NO: 19
SEQ ID NO: 13
deleted for clarity
36 mutations inserted to minimize recombination potential¹
11- nucleotide required cyclization sequence (CS)² SEQ ID NO: 2
genetic deletion
11-nucleotide 3' CS²

Fig. 9

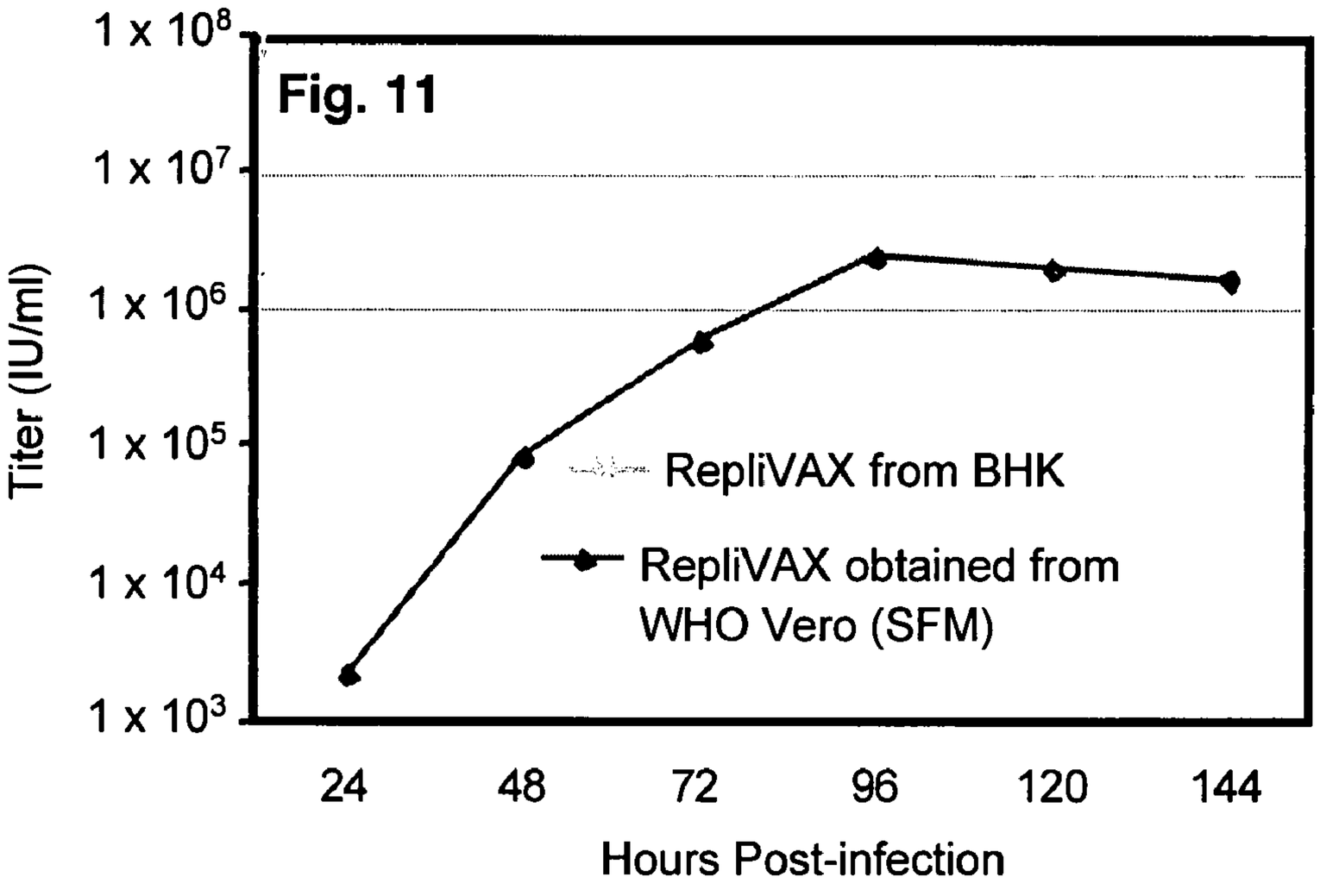


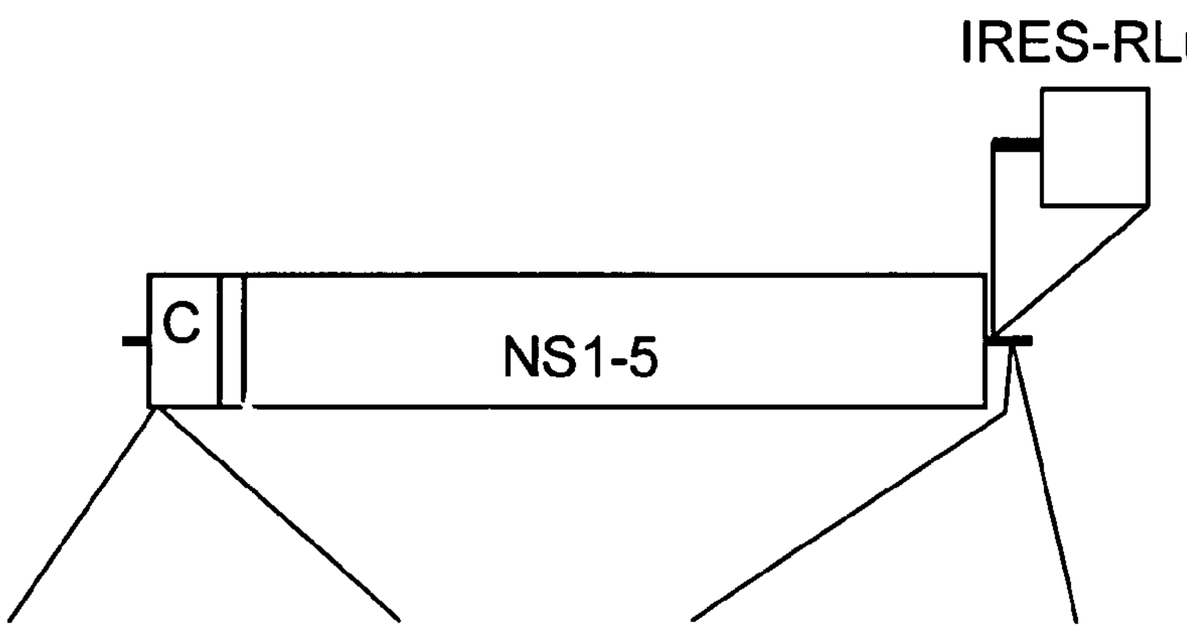
WN RepliVAX p0

Fig. 10

WN RepliVAX p10

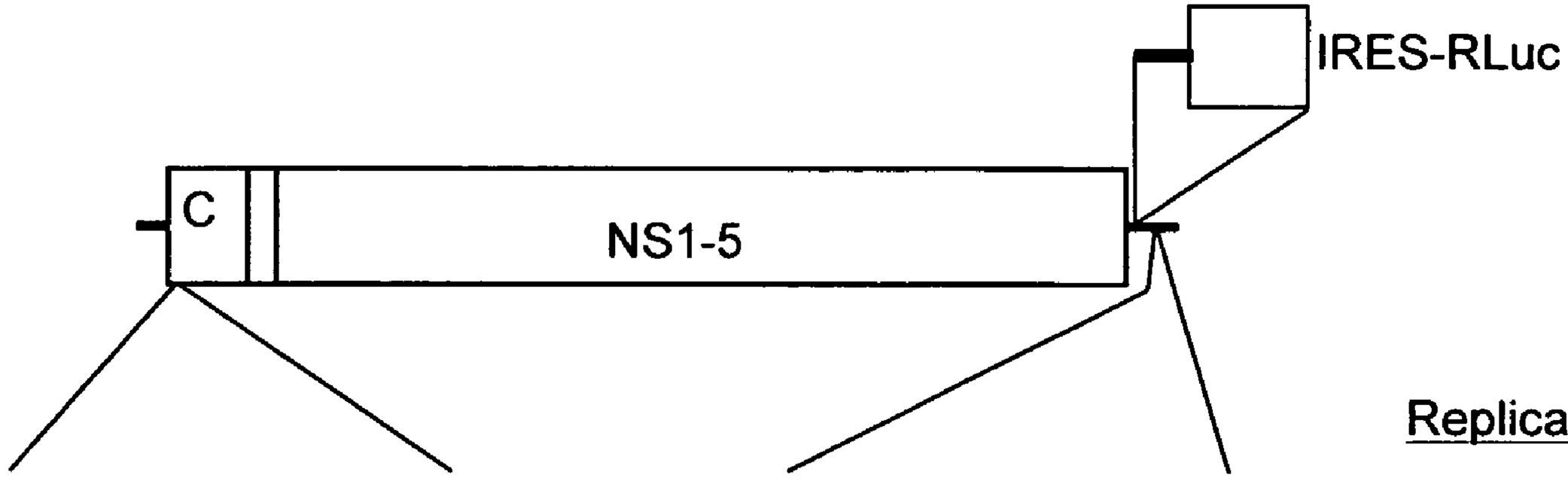
Fig. 11





			Replication level
WT	GUCAAUAUGCU(SEQ ID NO:2)	AGCAUAUUGAC(SEQ ID NO:30)	100
m7	CUCAAUAUGCU(SEQ ID NO:20)	AGCAUAUUGAG(SEQ ID NO:31)	2
m8	GACAAUAUGCU(SEQ ID NO:21)	AGCAUAUUGUC(SEQ ID NO:32)	3
m9	GUGAAUAUGCU(SEQ ID NO:22)	AGCAUAUUCAC(SEQ ID NO:33)	82
m10	GUCUAUAUGCU(SEQ ID NO:23)	AGCAUAUAGAC(SEQ ID NO:34)	173
m11	GUCAUUAUGCU(SEQ ID NO:24)	AGCAUAAUGAC(SEQ ID NO:35)	69
m12	GUCAAAAUGCU(SEQ ID NO:25)	AGCAUUUUGAC(SEQ ID NO:36)	69
m13	GUCAAUUUGCU(SEQ ID NO:26)	AGCAAAUUGAC(SEQ ID NO:37)	138
m14	GUCAAUAUCCU(SEQ ID NO:27)	AGGAUAUUGAC(SEQ ID NO:38)	24
m15	GUCAAUAUGGU(SEQ ID NO:28)	ACCAUAUUGAC(SEQ ID NO:39)	42
m16	GUCAAUAUGCA(SEQ ID NO:29)	UGCAUAUUGAC(SEQ ID NO:40)	69

Fig.12A



			Replication level
WT	GUCAAUAUGCU (SEQ ID NO:2)	AGCAUAUUGAC (SEQ ID NO:30)	100
m17	GUCUAUUUGCU (SEQ ID NO:41)	AGCAAAUAGAC (SEQ ID NO:42)	109
m17-5	GUCUAUUUGCU (SEQ ID NO:41)	AGCAUAUUGAC (SEQ ID NO:30)	<1*
m17-3	GUCAAUAUGCU (SEQ ID NO:2)	AGCAAAUAGAC (SEQ ID NO:42)	<1*
NC	Containing flame shift in NS5		

Fig. 12B

PSEUDOINFECTIOUS *FLAVIVIRUS* AND USES THEREOF

CROSS REFERENCE TO RELATED APPLICATION

This non-provisional application claims benefit of provisional application U.S. Ser. No. 60/777,189 filed on Feb. 27, 2006, now abandoned.

FEDERAL FUNDING LEGEND

This invention was produced in part using funds obtained through National Institute of Health grants (R01AI053135 and 1U54AI057156-010004). Consequently, the federal government has certain rights in this invention.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to the fields of molecular biology, virology and immunology. In general, the present invention discloses construction of replication-deficient viruses belonging to the Flaviviridae family and their use as vaccine in prevention of diseases caused by viruses belonging to this family. More specifically, the present invention provides replication-deficient flaviviruses or pseudoinfectious flaviviruses (PIV aka RepliVAX) and discloses its use as preventive vaccines against flavivirus-associated diseases.

2. Description of the Related Art

The Flavivirus genus of the Flaviviridae family contains a variety of important human and animal pathogens and have been classified into four distinct antigenic complexes based on differences in reactivity in immunological tests. Generally, the flaviviruses circulate between avian or mammalian amplifying hosts and mosquito or tick vectors.

The flavivirus genome is a single-stranded capped RNA of positive polarity lacking a 3' terminal poly(A) sequence. It encodes a single polypeptide that is co- and post-translationally processed into viral structural proteins, C, prM/M, and E, forming viral particles, and the nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, required for replication of viral genome and its packaging into infectious virions (Chambers, 1990). Virions contain a single copy of viral genomic RNA packaged into a C protein-containing nucleocapsid, surrounded by lipid envelope holding heterodimers of M and E proteins. In contrast to many other enveloped viruses, interaction between nucleocapsid and envelope spikes is not very specific and M/E-containing envelope can efficiently form around nucleocapsid derived from heterologous flavivirus, demonstrating limited level of homology in capsid sequence (Lorenz, 2002). Alternatively, expression of prM and E in the absence of C can lead to formation of subviral particles (SVPs), containing no RNA or C protein (Mason, 1991).

PrM/M-E cassettes producing subviral particles have been the basis of several vaccine candidates that are known in the art. These vaccine candidates include subunit (Konishi, 1992; 2001; 2002; Qiao, 2004), DNA (Phillpotts, 1996; Kochel, 1997; Schmaljohn, 1997; Colombage, 1998; Aberle, 1999; Konishi, 2000; Konishi, 2000; Kochel, 2000; Davis, 2001), and live-vectored (Mason, 1991; Konishi, 1992; Pincus, 1992; Fonseca, 1994; Pugachev, 1995; Colombage, 1998; Kanesa, 2000; Minke, 2004) vaccines. However, these vaccines have serious disadvantages. For instance, the subunit vaccines are safe to use but difficult to produce large quantities; the DNA vaccines are poorly immunogenic, and the viral vectored vaccines suffer from lack of potency in the presence of vector immunity.

Therefore, in spite of a great concern about flavivirus-associated diseases and continuing spread of the flaviviruses into the new areas, antiviral therapeutics have not yet been developed for these infections, and a very limited number of approved vaccines have been produced to-date. Inactivated viral vaccines (INVs) have been licensed to prevent tick-borne encephalitis (TBEV) and Japanese encephalitis (JEV). However, like other inactivated viral vaccines, these vaccines have low limited potency and require multiple vaccinations. Despite these drawbacks the Japanese encephalitis and tick-borne encephalitis INVs have an advantage of good safety records. The only licensed live-attenuated vaccine (LAV) for a flavivirus is the widely utilized live-attenuated vaccine based on the yellow fever virus (YFV) 17D strain that was developed by serial passaging of the wild type Asibi strain of yellow fever virus in chicken embryo tissues. Although this live-attenuated vaccine is considered very safe and effective, cases of yellow fever in vaccinees have been reported, including a recent case in a US military recruit (Gerasimon, 2005). Furthermore, this vaccine is not recommended for use in infants, pregnant women or the immunocompromised individuals due to adverse effects, including vaccine-associated encephalitis.

However, the development of the reverse genetics systems for flaviviruses has led to the production and designing of new types of live-attenuated vaccine, based on rational attenuation of these viruses. This new class of vaccines includes yellow fever virus 17D-based chimeras, in which the yellow fever virus prM-E-encoding genome fragment cassette has been replaced with the prM-E-cassette derived from heterologous flaviviruses (Chambers, 1999). Similar chimeric virus-based approach was applied for dengue- and TBE-based backbones (Pletnev, 2002; Huang, 2003). In most cases, chimeric flaviviruses demonstrate a highly attenuated phenotype and are capable of eliciting efficient protective immune response and protect against following infection with viruses, whose structural proteins are expressed by the chimeras (Monath, 2002). Effective vaccination with these chimeric vaccine candidates appears not to be prevented by pre-existing "vector" immunity (Monath, 2002), which has interfered with potency of recombinant viral vaccines based on other viral vectors. Further, although chimeric flaviviruses might provide a reasonably universal approach to producing new vaccines, there are concerns that the chimeras themselves might be pathogenic (Chambers, 1999) at least in the immunocompromised individuals, or that pathogenic chimeras might arise, since mutations have been detected during the process of propagation of these viruses (Pugachev, 2004).

Another promising direction in vaccine development is based on creating of irreparable deletions in flavivirus genome that make productive virus replication in the vaccinated host either a less efficient or an impossible event. In the latter case, viral genomes encoding the entire replicative machinery, but lacking, for instance, the C-coding region, can be delivered for in vivo immunization either as in vitro-synthesized RNA, capable of self-replication (Kofler, 2004; Aberle, 2005), or, probably, in DNA form (under control of the RNA polymerase II promoters or as in vitro-synthesized RNA, capable of self-replication (Kofler, 2004; Aberle, 2005). Direct immunization with in vitro synthesized defective RNA genomes, which specifies the production of SVPs in the absence of a complete viral replication cycle, has been demonstrated to be safe and effective in producing protective immunity (Kofler, 2004; Aberle, 2005). However, there may

be significant obstacles in producing an RNA-based vaccine candidate, due to synthesis, stability, and delivery issues.

Thus, prior art is deficient is deficient in a safe, potent and effective type of vaccine that can be used against diseases caused by infection with viruses belonging to the Flaviviridae family. The present invention fulfills this long-standing need and desire in the art.

SUMMARY OF THE INVENTION

In one embodiment of the present invention, there is provided a replication-deficient pseudoinfectious virus of Flaviviridae family. Such a replication-deficient pseudoinfectious virus comprises: a deletion in the nucleotide sequence encoding capsid (C) protein such that the deletion does not disrupt the RNA sequence required for genome cyclization, the signal sequence for prM protein that is required for the proper maturation of prM/M or a combination thereof, where the replication-deficient pseudoinfectious virus replicates only in cells expressing C protein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of the virus of the Flaviviridae family.

In another related embodiment of the present invention, there is provided a cell culture system expressing C protein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of the virus of the Flaviviridae family effective to enable propagation of the above-described replication-deficient pseudoinfectious virus of the Flaviviridae family under suitable conditions.

In yet another embodiment of the present invention, there is provided a method of producing the replication-deficient pseudoinfectious virus of the Flaviviridae family described above. Such a method comprises generating a replication-deficient pseudoinfectious virus of the Flaviviridae family that comprises deletion in the capsid gene such that the deletion does not disrupt the RNA sequence required for genome cyclization, the signal sequence for prM protein that is required for the proper maturation of prM/M or a combination thereof; generating a cell line that expresses C protein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of the virus of the Flaviviridae family, where the cell line provides high levels of the proteins of the Flaviviridae needed for propagation of the replication-deficient pseudoinfectious virus of the Flaviviridae family; and infecting the cell line with the pseudoinfectious virus of the Flaviviridae family, thereby producing the replication-deficient pseudoinfectious virus of the Flaviviridae family.

In another related embodiment of the present invention, there is provided a pharmaceutical composition, comprising the replication-deficient pseudoinfectious virus of the Flaviviridae family produced by the method described herein.

In a further related embodiment of the present invention, there is provided a method of protecting a subject from infections resulting from exposure to Flaviviridae. Such a method comprises administering to the subject an immunologically effective amount of the pharmaceutical composition produced by the method described herein, that elicits an immune response against the Flaviviridae in the subject, thereby protecting the subject from the infections.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic representation of flavivirus PIV replication in the cells producing C or all of the viral structural proteins for trans-complementation of the defect. Replication of PIVs in normal cells in vivo or in vitro leads to release SVPs having no nucleocapsid.

FIGS. **2**A-**2**C show that YFV C and YFV C, prM and E-expressing cell lines can complement replication of YF PIV. FIG. **2**A is a schematic representation of YFV and GFP-expressing YF PIV genome. FIG. **2**B is a schematic representation of VEEV replicons expressing Pac gene and YFV C with the signal peptide of prM (anchored C; VEErep/C1/Pac), or anchored C with 20 a. a. of prM (VEErep/C2/Pac), or all of the YFV structural proteins (VEErep/C-prM-E/Pac). FIG. **2**C shows the release of YF PIV by the cell lines transfected with in vitro-synthesized PIV genome. Media was replaced at the indicated time points, and titers of PIVs were determined. Arrows indicate time points when cells were subpassaged at a 1:5 ratio.

FIGS. **3**A-**3**B show growth curves of YF PIV on the packaging cell lines. BHK-21 cells containing VEErep/C2/Pac and VEErep/C-prM-E/Pac replicons were infected with YF PIV at indicated MOIs in infectious units per cell. At the indicated times, media was replaced and titers of released PIV were determined. Arrows indicate time points when cells were subpassaged at 1:5 ratio. FIG. **3**A shows growth curve at MOI 10 inf.u/cell and FIG. **3**B shows growth curve at MOI 0.1 inf.u/cell.

FIGS. **4**A-**4**C show that cells expressing codon-optimized C gene produced YF PIV. FIG. **4**A shows the nucleotide sequence of synthetic gene. The introduced mutations are indicated by lowercase letters (SEQ ID NO: 1). FIG. **4**B shows growth curves of YF PIV on the packaging cell lines. BHK-21 cells containing VEErep/C2/Pac, VEErep/C-prM-E/Pac, VEErep/C2opt/Pac and VEErep/Copt-prM-E/Pac replicons were infected with YF PIV at indicated MOIs in infectious units per cell. At the indicated times, media was replaced and titers of released PIV were determined. FIG. **4**C shows plaques developed in VEErep/C2opt/Pac-containing cell line by YFV and YF PIV after 4 days of incubation at 37° C.

FIGS. **5**A-**5**C show that WN PIV develops spreading infection in packaging cells. FIG. **5**A is a schematic representation of WN PIV genome and VEEV replicon expressing WNV structural genes. FIG. **5**B shows that WN PIV produced foci of WNV antigen-positive cells (revealed with an antibody to NS1-upon infection of BHK(VEErep/C*-E*-Pac) cells after 70 hours of incubation. FIG. **5**C shows the same WN PIV preparations produced only single infected cells (revealed at 70 hours post infection with the same tragacanth staining method used in FIG. **5**B) upon infection of Vero cell monolayers.

FIGS. **6**A-**6**C show detection of E protein upon release from cells infected with YF and WN PIVs. In FIG. **6**A, BHK-21 cells were infected with YF PIV at an MOI of 5 inf.u/cell. The released SVPs were harvested and purified by ultracentrifugation. Samples were resolved by SDS PAGE, transferred to filters, E protein was detected by D1-4G2 MAB. Media harvested from uninfected cells, lane 1; media harvested from the cells infected with YF PIV at 48 h post infection, lane 2; media harvested from the cells infected with YF PIVs at 72 h post infection, lane 3; YFV (2×10^7 PFU), lane 4. In FIG. **6**B, vero cells were infected with WN PIV for 24 hrs, and then portions of the clarified culture fluid (collected before any cell lysis was detected), were resolved by SDS PAGE, transferred to filters, and reacted with an E-specific MAB (7H2; Bioreliance). Reaction of the same samples with polyclonal sera failed to reveal any cell-associated non-structural proteins in this preparation (results not shown) confirming that the E protein was actively secreted. Sample of WNV, lane 1; media harvested from uninfected cells, lane 2; media harvested from the cells infected with WN PIV at 48 h post

infection, lane 3. In FIG. **6**C, a western blot showing E protein content of fractions prepared form a sucrose density gradient obtained from SVPs harvested from normal (non-packaging) BHK cells electroporated with YFV PIV RNA. The peak of E protein reactivity (at 32% sucrose) corresponded to the density of SVPs and in agreement with this fact, migrated more slowly than YFV run in a side-by-side analyses (42%).

FIGS. **7**A-**7**F show schematic representation of plasmids used for Yellow fever (YF) and West Nile (WN) pseudoinfectious virus (PIV) production. FIG. **7**A shows pYFPIV, FIG. **7**B shows pWNPIV, FIG. **7**C shows pVEErep/C1/Pac, FIG. **7**D shows pVEErep/C2/Pac, FIG. **7**E shows pVEErep/C3/PAc, FIG. **7**F shows pVEErep/C*-E*-Pac.

FIGS. **8**A-**8**V show the sequences of the plasmids used herein. FIGS. **8**A-**8**D shows sequence of pYFPIV (SEQ ID NO: 6), FIGS. **8**E-**8**H shows sequence of pVEErep/C1/Pac (SEQ ID NO: 7), FIGS. **8**I-**8**K shows sequence of pVEErep/C2/Pac (SEQ ID NO: 8), FIGS. **8**L-**8**O shows sequence of pVEErep/C-prM-E/Pac (SEQ ID NO: 9), FIGS. **8**P-**8**R shows sequence of pVEErep/C2opt/pac (SEQ ID NO: 10), FIGS. **8**S-**8**V shows sequence of pVEErep/Copt-prM-E/Pac (SEQ ID NO:11).

FIG. **9** shows a schematic representation of overlapping regions of RepliVAX and the VEE replicon used to provide C in trans. [1]Thirty-six mutations were inserted into the VEErep/pac-Ubi-C* to minimize homologous recombination with the fragment of C encoded by the RepliVAX genome. [2]Position of 5' and 3' CS sequences in the RepliVAX genome.

FIG. **10** shows side by side comparison of infectious foci produced in the C-expressing cell line {BHK(VEErep/Pac-Ubi-C*)} by WN RepliVAX at passage 0 (from electroporation) and passage 10 reveals that better-growing variants are readily selected.

FIG. **11** shows titration of RepliVAX PIV produced in WHO-certified Vero cells containing a C-expression cassette (VEErep/Pac-Ubi-C*). Although the resulting PIV is of a slightly lower titer than that produced in BHK cells, the Vero cells multiple harvests of high titer PIV, which is not possible with BHK cells.

FIGS. **12**A-**12**B show cyclization mutants. FIG. **12**A shows replication of WNV/IRES-RLuc replicon with single-base, matching CS mutations demonstrates that some single-base mutations replicate at WT levels. Left part of panel shows the test genome above the 5' and 3'CS sequences. Right side shows replication levels detected using Rluc reporter, as a percentage of the WT replication levels. Underlined bases denotes mutated bases. FIG. **12**B shows replication of WNV/IRES-RLuc replicon with matching the double-base changes (m17) derived by combining m10 and m13 (Panel A), compared to replication levels detected with mutants that combine the WT and mutated CS in either possible format, along with a mutant designed to produce an inactive polymerase (negative control). Left part of panel shows the test genome above the 5' and 3' CS sequences. Right side shows replication levels detected using Rluc reporter, as a percentage of the WT replication levels. Underlined bases denotes mutated bases. * denotes no replication detected.

DETAILED DESCRIPTION OF THE INVENTION

Safe and effective vaccines have only been produced for a handful of diseases caused by flaviviruses. The classical inactivated viral vaccine (INV) and live-attenuated vaccine (LAV) methods that have been used to produce vaccines to YF, JE, and TBEV have not yet yielded licensed products to prevent diseases caused by other flaviviruses, notably dengue and West Nile encephalitis (WNE). There remain safety concerns about existing LAVs (residual virulence or reversion to virulence) and INV products (reactogenicity due to antigen load and adventitious antigens). Additionally, INVs usually require multiple vaccinations. Further, both types of vaccines are subject to production concerns, including the need to avoid reversion to virulence during propagation of live-attenuated vaccine, and due to the amounts of material needed to produce strong immune responses to the inactivated viral vaccine products and the need for high containment facilities to propagate the virulent viruses used to produce INV products. Although there are promising candidates for new types of flavivirus vaccines, the road to their development will need to overcome these problems.

The present invention in general, is drawn to construction and utilization of replication-deficient pseudoinfective viruses belonging to the Flaviviridae family. In this regard, the present invention describes the development a new type of replication-deficient flaviviruses also referred to as RepliVAX that combines the safety of inactivated vaccines with the efficacy and scalability of live attenuated vaccines. These flaviviruses also identified as pseudo-infectious viruses (PIVs) in the present invention contain genetically engineered flavivirus genomes with a deletion of most of the capsid (C)-encoding region, thereby preventing this genome from producing infectious progeny in normal cell lines or vaccinated animals. However, these pseudo-infectious viruses can be propagated in cell lines expressing either C, or a C-prM-E cassette, where they replicate to high levels. Thus, these pseudoinfectious flaviviruses cannot develop spreading infection in normal cells in vitro or in vivo due to lack of trans-complementation by C protein, and therefore are incapable of causing disease in animals.

In contrast to the vaccines and the methods to generate these vaccines that are known in the art, the present invention provides a system for industrial-scale production of pseudo-infectious flaviviruses that would make such vaccines cheaper to produce than inactivated vaccines at the same time making it safer to use than live-attenuated vaccines. It does so by providing a new type of recombinant vaccine that is capable of only single round of replication in the immunized animals or humans leading to release of subviral particles (SVPs) lacking the genetic material but serving as efficient immunogens.

The present invention has demonstrated that pseudoinfectious flaviviruses can be generated for either yellow fever virus (YFV) or West Nile virus (WNV). Based on this, the present invention contemplates that the method described herein could be broadly applicable to the development of vaccines against other flaviviruses. Further, infection of normal cell lines with such pseudoinfectious flaviviruses produced SVPs that lacked nucleocapsid and a genetic material. The pseudoinfectious flaviviruses described herein demonstrated inability to cause any disease and thus were safe. Additionally, these pseudoinfectious flaviviruses were immunogenic in mice due to competency for single round of replication and release of SVPs, presenting viral antigens. WN PIVs also protected mice from a lethal encephalitis following challenge with WNV.

The PIVs described herein could be produced in a manner that allows for high-yield production in cell culture, and inability to cause disease in animals. These products could be delivered to animals where their defective replication process prevents spread and disease, but permitted the production of SVPs, a flavivirus subunit immunogen that has been shown to be effective in eliciting an efficacious immune response against disease caused by several flaviviruses.

The present invention also demonstrated that the pseudoinfectious flaviviruses approach could be applied to two distantly related mosquito-borne flaviviruses. The applicability of a similar technology to the development of RNA-based vaccines for a tick-borne flavivirus (Kofler, 2004) indicates that the PIV-based technology will be applicable to more distantly related flaviviruses. Additionally, the work with TBEV RNA-based vaccines indicates that in addition to antibody responses to the SVPs (similar to that described herein), the introduction of replicationally active flavivirus genomes into the cells of the vaccinated hosts will produce T-cell responses as well (Kofler, 2004; Aberle, 2005). Although the T cell responses were not measured herein, it is contemplated that the PIVs are capable of inducing T cell response that mimics those produced by viral infection.

Although the PIV vaccines described herein rely on the same flavivirus replication and SVP production strategy that was utilized by the RNA-based vaccines prepared for TBEV (Kofler, 2004; Aberle, 2005), these PIV vaccines do not require gene-gun delivery to animals, can be readily grown in cell cultures, and can subjected to the same types of stabilization and storage (freeze drying) conditions currently being applied to the commercially produced YFV 17D vaccine, thus providing a scalable, storable, and marketable vaccine product. Preliminary studies on stability of WN RepliVAX have shown that freeze-dried preparations show no detectable loss in titer when stored for 30 days at 4 C.

To develop the high-level growth conditions required for efficient trans-complementation (and hence yield) of pseudoinfectious flaviviruses, the present invention utilized cells expressing high levels of C (or C-prM-E) from VEEV replicons. VEEV replicons are less cytopathic than the replicons derived from other alphaviruses and readily establish persistent replications in some cell lines of vertebrate and insect origin. This system appears to be suitable for production of pseudoinfectious flaviviruses, since i) VEEV replicons are highly efficient in synthesis of heterologous proteins and, in the present invention synthesized C to the level required for flavivirus genome incapsidation. ii) VEEV replicons do not detectably interfere with flavivirus replication (Petrakova, 2005). Moreover, VEE replicons and the YF PIV genomes can replicate together in BHK-21 cells without causing CPE. iii) VEEV replicons can be packaged at high-titers into VEE virions that can be used for rapid establishment of the packaging cell lines producing flavivirus structural protein(s).

Furthermore, examination of the effect of context of C expression on yield of PIV indicated that the packaging cells expressing anchored form of C with an additional 20 a.a. of prM produced more particles than cells expressing anchored C alone, suggesting the importance of the proper sequence of processing events in virions formation. The basis for the enhanced packaging efficiency by the construct containing the first 20 amino acids of prM is unclear but this phenomenon might be explained by a requirement of specific order of cleavage at the two nearby cleavage sites (NS2B/NS3- and signal peptidase-specific) (Yamshchikov and Compans, 1994) and/or differences in distribution/stability of C protein products in these two different contexts. In addition, it was observed that co-expression of C with prM and E (VEErep/C-prM-E/Pac) caused only minor increase in PIV yield compared to VEErep/C2/Pac, which expressed anchor C with the fragment of prM.

When the codon-optimization of the VEEV replicon-encoded C genes was examined to determine if this alteration of the C gene sequence enhanced yield of PIV, it did not reveal a strong difference in YFV PIV release from the cells not expressing a codon-optimized C gene. This observation suggested that even with the non-optimized gene VEEV replicons appear to produce C at a saturating level. These results were consistent with other studies demonstrating that the cell lines that expressed VEEV replicons encoding the WNV C-E cassette produced level of E greater than those detected in WNV-infected cells. Despite the inability of the trans-expressed optimized C gene to increase yield of YF PIV, the cells harboring the VEEV replicon expressing Copt developed CPE and produced plaques when infected with YF PIV. This made a PIV infection in the Copt cells even more similar to infection developed by replication-competent virus. An additional advantage of the use of VEEV replicons encoding a YFV Copt gene in pseudoinfectious flavivirus production was the level of safety, since the changes in the codons reduced the chance of homologous recombination with the pseudoinfectious flavivirus genome. Furthermore, the Copt gene was also altered in its cyclization sequence (as described herein for the WNV C coding region in the BHK(VEErep/C*-E*-Pac) cells), to reduce the chance of the recombination producing a replicationally active C-encoding flavivirus. To date, neither the WN nor YF PIV systems described herein have produced replicationally active flaviviruses that could be detected in either cell culture, or in highly susceptible animals. Additionally, in vivo experiments demonstrated that both YF and WN PIVs were safe and could not cause any disease even after i.c. inoculation of 3- to 4-day-old mice with the highest dose of the PIVs. Nevertheless, WN PIVs were capable of inducing high levels of neutralizing antibodies and protected mice against infection with replication competent WNV.

Furthermore, Hepatitis C ranks with AIDS as a major infectious cause of morbidity and mortality for which no vaccine is currently available. In Japan and Korea, HCV now exceeds hepatitis B in contributing to the development of hepatocellular carcinoma, one of the most common types of cancer and a common mode of death due to liver disease. This pattern is likely to become increasingly common in other Asian countries and elsewhere in the developing world, due to the increasing prevalence of HCV coupled with effective immunization against hepatitis B. In some communities in Egypt and elsewhere, the prevalence of hepatitis C infection is spectacularly high, likely due at least in part to traditional health care practices and/or the introduction of dangerous Western technologies in the past (e.g., needle-borne transmission of the virus during public health campaigns directed against schistosomiasis).

In many developing countries, where rates of liver cancer and cirrhosis are high, there is little effective control of hepatitis C during blood transfusion. Hepatitis C is also a major public health problem within the United States, where there are approximately 4 million carriers of HCV, many of whom are at risk of death due to end-stage liver disease or liver cancer. Currently it is estimated that there are between 8,000 and 10,000 deaths annually due to hepatitis C in the United States. This number is likely to triple over the next 10-20 years, potentially exceeding the number of deaths due to AIDS, in the absence of new therapeutic or preventive measures.

Yet, no vaccine is available for prevention of this infection, and efforts (both national and international) to develop a vaccine are severely limited due to perceived technical difficulties, little interest in vaccine development generally on the part of big pharma, and the inertia of major funding agencies. And, as with many infectious diseases, it is the disadvantaged who are at greatest risk of serious liver disease or death due to hepatitis C.

To date attempts to create an effective vaccine against HCV infection have been unsuccessful. However, within last few years, the HCV field started to rapidly develop, and now this virus replicates in tissue culture to reasonably high titers, approaching 10^6 inf.u/ml. There is a number of obvious similarities between the HCV genome and the genomes of other flaviviruses, like YF, JEV, TBE and others. Therefore, the strategy of designing replication-deficient flaviviruses can be applied not only to the members of the Flavivirus genus, but to Hepacivirus genus (that include HCV) as well. The HCV capsid protein can produced by recombinant alphavirus replicons (based on SINV, VEEV EEEV and others) in a number of cell lines, including Huh-7 and Huh-7.5 cells that are currently known to be susceptible to HCV infection. Replication-deficient HCV genomes, lacking the capsid gene can be transfected into the capsid-producing cell lines and will be packaged into infectious, capsid-containing particles. The successive rounds of infection required for the large-scale production, can be performed on these cells as well. However, in vivo, in the naïve hepatocytes (and possibly other cell types), the HCV genomes lacking the complete capsid gene or no capsid gene at all, will produce only the nonstructural viral proteins, and glycoproteins E1 and E2. These proteins will be presented to immune system i) after proteasome degradation; ii) on the cell surface and iii) in the form of virus-like particles with E1- and E2-containing envelope. Capsid deficiency will make virus incapable of spreading, and thus limited to the cells infected by the vaccinating dose.

In summary, the present invention demonstrated that capsid-deficient, pseudoinfectious flaviviruses i) could produce a spreading infection in the cell lines expressing capsid or all of the flavivirus structural genes; ii) PIVs were incapable of producing spreading infection in normal cells, (iii) PIVs produced E protein containing SVPs when they infected normal cells; (iv) PIVs displayed a high level of safety in the animals; (v) PIVs protected the mice from subsequent flavivirus infection. Taken together, the present invention demonstrated that flavivirus PIVs might be a safe, potent, and efficacious platform for development of vaccines against flavivirus infections and infections caused by viruses similar to Favivirus.

The present invention is directed to a replication-deficient pseudoinfectious Flaviviridae, comprising: a deletion in the nucleotide sequence encoding capsid (C) protein such that the deletion does not disrupt the RNA sequence required for genome cyclization, the signal sequence for prM protein that is required for the proper maturation of prM/M or a combination thereof, where the Flaviviridae replicate only in cells expressing Cprotein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of a virus of the Flaviviridae family. Generally, the Flaviviridae comprises a virus belonging to the genus flavivirus, Hepacivirus or Pestivirus or other chimeras of said viruses created by exchanging the prM-E cassettes of other viruses with the prM-E cassettes of the pseudoinfectious Flaviviridae. The examples of the viruses belonging to the genus Flavivirus are not limited to but may include yellow fever virus, West Nile virus, dengue virus, tick-borne encephalitis virus, Saint Louis encephalitis virus, Japanese encephalitis virus, Murray Valley encephalitis virus. Furthermore, the example of the virus belonging to the genus Hepacivirus includes but is not limited to Hepatitis C virus and those belonging to the genus Pestivirus include but are not limited to Bovine virus diarrhea, a swine fever virus or a hog cholera virus.

In case of flavivirus, the nucleotide sequence encoding the C protein of the Flavivirus that is deleted may encode amino acids 26 to 100 or a combination of amino acids within amino acids 26 to 100 of the C protein. Such combinations may include but are not limited to amino acids 26-93, 31-100 or 31-93. One of ordinary skill in the art can use the same guidelines to delete nucleotide sequence of C protein from other viruses belonging to the Flaviviridae family or other viruses having the same genetic makeup as these viruses. In general and applicable to all the viruses, the deleted gene is replaced by a gene encoding a marker protein or an antigen. The example of a marker protein may include but is not limited to a green fluorescent protein.

The present invention is also directed to a cell culture system expressing C protein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of a virus of the Flaviviridae family, effective to enable propagation of the above-described replication-deficient Flaviviridae under suitable conditions. For this purpose, the cells expressing wild type or mutated proteins of the Flaviviridae may be generated using genetically engineered replicons derived from viral vectors.

In general, the gene encoding the protein(s) of the virus of the virus Flaviviridae family in the replicon is replaced by a codon-optimized version of the gene encoding the protein(s) of the virus such that the replacement reduces the ability of the cell line-encoded genes to recombine with the genome of the pseudoinfectious virus of the Flaviviridae family and/or increases the production of the pseudoinfectious virus of the Flaviviridae family.

For instance, such replicons may express a C protein that comprises mutations in at least 36 nucleotide positions of the gene encoding C protein of the virus of the Flaviviridae family. The replicon may express a C protein in the replicon that comprises unnatural cyclization sequences such that presence of the cyclization sequences reduces the chances of productive recombination of the replication-deficient pseudoinfective virus with natural viruses. Further, the replicon may express proteins comprising altered nucleotide sequences encoding truncated C-prM junction such that presence of such altered sequences enhances yield of the replication-deficient pseudoinfective virus in cell culture, prM/E containing SVP yield in vivo or a combination thereof.

Furthermore, the replicons expressing the proteins of Flaviviridae are introduced into the cells by transfection with in vitro synthesized replicon RNAs, by transfection with plasmid DNAs designed to synthesize functional alphaviral replicons from cellular RNA-polymerase II-specific promoters or by infection with alphaviral replicons packaged inside the alphaviral structural proteins. The viral vectors used herein may be alphaviruses. Representative examples of such alphaviruses are not limited but may include Venezuelan Equine Encephalitis Virus (VEEV), Sindbis virus, Eastern Equine Encephalitis virus (EEEV), Western Equine Encephalitis virus (WEEV) or Ross River virus.

The present invention is further directed to a method of producing a replication-deficient pseudoinfectious virus of the Flaviviridae family, comprising; generating a replication-deficient pseudoinfectious virus of the Flaviviridae family that comprises a deletion in the capsid gene such that the deletion does not disrupt the RNA sequence required for genome cyclization, the signal sequence for prM protein that is required for the proper maturation of prM/M or a combination thereof; generating a cell line that expresses C protein or C, prM, envelope protein, mutated C protein, mutated prM, mutated envelope protein or combinations thereof of a virus of the Flaviviridae family, where the cell line provides high levels of the proteins needed for propagation of the replication-deficient pseudoinfectious virus of the Flaviviridae fam-

ily; and infecting the cell line with the pseudoinfectious virus of the Flaviviridae family, thereby producing the replication-deficient pseudoinfectious virus of the Flaviviridae family. All other aspects regarding the types of viruses, the position of deletions in the capsid gene, the method of generation of the cell line expressing the mutated and wild type proteins of the Flaviviridae, the type of replicons and the mutations within the replicons and the modifications in the gene encoding the mutated and wild type proteins of the Flaviviridae in the replicons are the same as discussed supra.

The present invention is also directed to a pharmaceutical composition, comprising the replication-deficient pseudoinfectious virus of the Flaviviridae family produced by the method described supra. The present invention is further directed to a method of protecting a subject from infections resulting from exposure to Flaviviridae, comprising administering to the subject an immunologically effective amount of the pharmaceutical composition described herein, where the composition elicits an immune response against the Fiaviviridae in the subject, thereby protecting the subject from the infections. Such a composition may be administered via intraperitoneal, intradermal, subcutaneous, intramuscular, oral, or intranasal route. Furthermore, the subject benefiting from use of this composition may be a human, or an animal.

As used herein, the term, "a" or "an" may mean one or more. As used herein in the claim(s), when used in conjunction with the word "comprising", the words "a" or "an" may mean one or more than one. As used herein "another" or "other" may mean at least a second or more of the same or different claim element or components thereof. As used herein, the term, "Flaviviridae" includes the genera Flavivirus, Hepacivirus and Pestivirus. The examples of virus belonging to the genus Flavivirus include but are not limited to yellow fever virus, West Nile virus, dengue virusm a tick borne encephalitis virusm a Saint Louis encephalitis virus, a Japanese encephalitis virus or a Murray Valley encephalitis virus, Similarly, the example of virus belonging to the genus Hepacivirus includes but is not limited to Hepatitis C virus and those belonging to the genus Pestivirus include but are not limited to Bovine virus diarrhea, a swine fever virus or a hog cholera virus.

Furthermore, although the present invention discloses the construction and utility of a replication-deficient pseudoinfectious Flaviviridae belonging to the genus Flavivirus, one of ordinary skill in the art can use the same guidelines to construct chimeras comprising other viruses belonging to the Flaviviridae or to construct chimeras by exchanging the prM-E cassettes of viruses within the Flaviviridae or other similar viruses and the viruses within the Falviviridae.

The pharmaceutical compositions comprising the pseudoinfectious viruses belonging to the Flaviviridae family discussed herein may be administered concurrently or sequentially with each other or with other pharmaceutical composition(s). The effect of co-administration of such compositions is to protect an individual from the infections caused by such viruses and other vaccine-treatable disease. The composition described herein, the other pharmaceutical composition, or combination thereof can be administered independently, either systemically or locally, by any method standard in the art, for example, subcutaneously, intravenously, parenterally, intraperitoneally, intradermally, intramuscularly, topically, enterally, rectally, nasally, buccally, vaginally or by inhalation spray, by drug pump or contained within transdermal patch or an implant. Dosage formulations of the composition described herein may comprise conventional non-toxic, physiologically or pharmaceutically acceptable carriers or vehicles suitable for the method of administration and are well known to an individual having ordinary skill in this art.

The composition described herein, the other pharmaceutical composition or combination thereof may be administered independently one or more times to achieve, maintain or improve upon a therapeutic effect. It is well within the skill of an artisan to determine dosage or whether a suitable dosage of either or both of the compositions comprises a single administered dose or multiple administered doses. An appropriate dosage depends on the subject's health, the protection of the individual from flaviviral infections, the route of administration and the formulation used.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion. One skilled in the art will appreciate readily that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those objects, ends and advantages inherent herein. Changes therein and other uses which are encompassed within the spirit of the invention as defined by the scope of the claims will occur to those skilled in the art.

Example 1

Cell Cultures

The BHK-21 cells were provided by Paul Olivo (Washington University, St. Louis, Mo.). They were maintained at 37° C. in alpha minimum essential medium (aMEM) supplemented with 10% fetal bovine serum (FBS) and vitamins. WHO-certified Vero cells, originally prepared for use in human vaccine manufacture were provided by Dr. Steve Whitehead of the NIH. Vero cells were maintained in MEM containing 6% FBS.

Example 2

Plasmid Constructs

Standard recombinant DNA techniques were used for all plasmid constructions. A schematic representation of the plasmids used are shown in FIGS. **7**A-**7**F. Maps and sequences are shown in FIGS. **8**A-**8**F. The parental low-copy number plasmid pACNR/FLYF-17Dx containing infectious cDNA of YFV 17D strain genome was described elsewhere (Bredenbeek et al., 2003) and provided by Dr. Charles M. Rice (Rockefeller University, New York, N.Y.). pYFPIV contained defective YFV genome (YF PIV), in which fragment encoding amino acid. 26-93 of YF capsid gene was replaced by codon-optimized GFP gene derived from pEGFP-N1 (Clontech). The WN PIV genome (pWNPIV) was derived from a Texas 2002 infectious cDNA (Rossi et al., 2005), by fusion of codon 30 of C to codon 101 of C (see FIG. **5**A). The plasmids pVEErep/C1/Pac, pVEErep/C2/Pac and pVEErep/C-prM-E/Pac (FIG. **2**A) encoded double subgenomic VEEV replicons, in which the first subgenomic promoter was driving transcription of the RNAs containing 5'UTR of VEEV 26S RNA followed by sequences, corresponding to nt 119-481, 119-541 and 119-2452 of YFV genome, respectively. The second subgenomic promoter was driving the expression of puromycin acetyltransferase (Pac) gene, whose product was making cells resistant to translational arrest caused by puromycin (Pur). Non-cytopathic VEEV replicons expressing the C-prM-E cassette of WNV {derived from a Sindbis virus replicon (Scholle et al., 2004)} fused to the Pac gene

(designated pVEErep/C*-E*-Pac) was created from a VEE non-cytopathic replicon (Petrakova et al., 2005); E-coding sequence was fused with Pac gene through a linker consisting of the first 9 codons of NS1 and the last 25 codons of NS2B, followed by 2 codons of NS3 fused directly to FMDV 2A (see FIG. **5**A). The codon-optimized sequence encoding YFV 17D capsid and first 20 amino acid of prM was designed using the codon frequency data described elsewhere (Haas et al., 1996). This gene was synthesized by PCR from the overlapping synthetic oligonucleotides. The amplified fragment was sequenced before cloning into expression cassettes VEErep/C2opt/Pac and VEErep/Copt-prM-E/Pac. The latter replicons had essentially the same design as pVEErep/C2/Pac and pVEErep/C-prM-E/Pac, but contained codon-optimized sequence presented in FIG. **4**A.

Additionally, a chimeric WN-RepliVAX expressing the JEV prM-E has also been generated. This was constructed by substituting the prM and E genes of Nakayama strain of JEV in A RepliVAX encoding the WNV genome. The gene exchange was achieved by direct fusion of the last codon of the truncated WNV C protein to the first codon of the prM coding sequence of JEV (Nakayama strain). The same fusion strategy was employed at the 3' end of the cassette, with the final codon of the JEV E protein fused directly to the first codon of NS1 of WNV. These fusions were introduced into a BAC plasmid encoding the entire WN RepliVAX cDNA bounded by a T7 promoter and a ribozyme, and RNA recovered from this BAC DNA was introduced into BHK(VEErep/Pac-Ubi-C*) cells. The resulting RepliVAX (designated JE RepliVAX) formed spreading infectious foci on BHK (VEErep/Pac-Ubi-C*). As for WN RepliVAX, the foci formed on this cell line are smaller than those produced by a fully infectious WNV-JEV chimera. JE RepliVAX grows to titers approximately 10 times lower than WN RepliVAX, achieving titers of over 10^6 U/ml in BHK(VEErep/Pac-Ubi-C*). As expected, JE RepliVAX reacts with JE-specific MAbs, and it is anticipated that like chimeric flaviviruses, JE RepliVAX will induce high levels of JEV-neutralizing antibodies, and protect against JE.

Example 3

RNA Transciptions

Plasmids were purified by centrifugation in CsCl gradients. Before the transcription reaction, the plasmids were linearized by XhoI (for pYFPIV) or MhuI (for VEE replicon and VEE helper encoding plasmids) or SwaI (for pWNPIV). RNAs were synthesized by SP6 or T7 RNA polymerase in the presence of cap analog. The yield and integrity of transcripts were analyzed by gel electrophoresis under non-denaturing conditions. Aliquots of transcription reactions were used for electroporation without additional purification.

Example 4

RNA Transfections and PIV Replication Analysis

Electroporation of BHK-21 cells was performed under previously described conditions (Liljestrom et al., 1991). For establishing packaging cell cultures, Pur was added to the media to a concentration of 10 g/ml 24 h post electroporation of the VEEV replicons. Transfection of in vitro-synthesized YF PIV genome was performed 5 days later, when replicon-containing cells resumed efficient growth. Samples were harvested at the time points indicated in the figures by replacing the media with the same media, containing Pur. In many experiments, PIV-secreting cells were split upon reaching the confluency.

VEEV replicons were packaged into VEEV infectious virions by co-electroporation of the in virto synthesized replicon and 2 helper RNAs (Volkova et al., 2006) into BHK-21 cells. Replicon-containing viral particles were harvested 24 h post transfection and used for infecting of naïve BHK-21 cells, followed by Pur selection. In the case of WN PIV, the in vitro-synthesized PIV RNA was transfected into BHK cells containing VEErep/C*-E*-Pac replicon expressing WN C, prM and E and Pac [BHK(VEErep/C*-E*-PAC) cells]. THE scheme of the VEErep/C*-E*-PAC genome is presented in FIG. **5**A. Harvested PIVs were passaged on these cells using standard methods (Rossi et al., 2005).

Example 5

Measuring the Titers of YF PIV

For measuring the titers of released YF PIV, BHK-21 cells were seeded into six-well Costar dishes at a concentration of 5×10^5 cells/well. Four hours later, cells were infected with different dilutions of packaged replicons, and after 1 h incubation at 37° C. in an 5% CO_2 incubator, they were overlaid with 2 ml of MEM supplemented with 10% FBS. The numbers of infected cells were estimated by counting GFP-positive cells under an inverted UV microscope. The fraction of infected cells from the seed quantity was determined via counting of fluorescence-producing cells in a defined area of microscopic field. Counts for 5 different fields were averaged and recalculated for the titer corresponding to each serial dilution.

In the later experiments, titers were also measured by plaque assay on the monolayers of BHK-21 cells, carrying VEErep/Copt-prM-E/Pac replicon, using previously described conditions (Lemm et al., 1990), except cells were incubated under agarose for plaque development for 5 days, then fixed by 2.5% formaldehyde and stained with crystal violet.

Example 6

Passaging of YF PIVs

Packaging cell lines were established either by transfection of the in vitro-synthesized VEEV replicon RNAs or by infecting cells with the same replicons packaged into VEEV structural proteins at a multiplicity of infection (MOI) of 10 inf.u/cell. After Pur selection, they were infected with YF PIV at different MOIs. Samples were harvested at the time points indicated in the figures by replacing the media.

Example 7

Analysis of YF SVP Production

BHK-21 cells were seeded at a concentration of 2×10^6 per 100-mm dish. After 4 h incubation at 37° C., cells were infected with YF PIV at an MOI of 10 inf.u/cell, and then incubated for 24 h in 10 ml of MEM supplemented with 10% FBS. Then the medium was replaced by 10 ml of serum-free medium VP-SF (Invitrogen) that was replaced every 24 h to analyze SVP release. The collected VP-SF samples were clarified by low-speed centrifugation (5,000 r.p.m, 10 min, 4° C.), and then concentrated by ultracentrifugation through 2 ml of 10% sucrose, prepared on PBS, in SW-41 rotor at

39,000 r.p.m, 4° C. for 6 h. Pellet material was dissolved in the loading buffer for SDS-polyacrylamide gel electrophoresis, lacking b-mercaptoethanol (to preserve binding to D1-4G2 MAB) and further analyzed by Western blotting. After protein transfer, the nitrocellulose membranes were processed by D1-4G2 MAB, and horseradish peroxidase (HRP)-conjugated secondary donkey anti-mouse antibodies purchased from Santa Cruz Biotechology. HRP was detected using the Western Blotting Luminol Reagent according to the manufacurer's recommendations (Santa Cruz Biotechnology). To obtain positive control sample, YFV (2×10^8 PFU) was subjected to ultracentrifugation through 10% sucrose cushion as described above for SVPs.

For sucrose density gradient analysis of YFV SVPs, BHK-21 cells were electroporated with the in vitro synthesized YF PIV genome RNA. At 24 hours post-transfection, the complete MEM was replaced by VP-SF medium, which was harvested 24 hours later. At this time, more than 95% of the cells were GFP-positive and did not exhibit any signs of CPE. The harvested sample was clarified by low-speed centrifugation (5000 rpm, 10 min, 4° C.) and then concentrated by overnight centrifugation in a SW-28 rotor at 25,000 rpm, 4° C. The resulting pellet was suspended in TN buffer (10 nm Tris HCl (pH 7.5), 100 mM NaCl, 0.1% BSA) and further analysis was performed as described (Schalich et al., 1996).

Briefly, 0.5 ml samples were loaded in to the discontinuous sucrose gradient (1.5 ml of 50%, 1.5 ml of 35% and 1.5 ml of 10% sucrose prepared in PBS buffer). Centrifugation was performed in SW-55 rotor at 45,000 rpm at 4° C. for 1 h in Optima MAX Ultracentrifuge (Beckman). Pellets were dissolved in the loading buffer for SDS polyacrylamide gel electrophoresis, lacking b-mercaptoethanol (to preserve binding to D1-4G2 MAB) and further analyzed by Western blotting. After protein transfer, the nitrocellulose membranes were processed by D1-4G2 MAB and horseradish peroxidase (HRP)-conjugated secondary donkey anti-mouse antibodies purchased from Santa Cruz Biotechnology. HRP was detected using Western Blotting Luminol Reagebbr according to the manufacturer's recommendation (SantaCruz Biotechnology). Side by side gradient analyses were performed with YFV (2×10^8 PFU), subjected to the same procedures as described above for YFV-PIV derived SVPs.

Example 8

Analyses of WN PIV

Titers of WN PIV were determined by infecting Vero cell monolayers with serial dilutions of virus, and then fixing 24 hours later and immunohistochemically staining with a polyclonal hyperimmune mouse ascite fluid specific for WNV, as previously described (Rossi et al., 2005). Infected cells were enumerated and used to determine the titer. To evaluate the ability of WN PIV for foci formation on Vero cells or the BHK(VEErep/C*-E*-PAC) cells, monolayers were infected with dilutions of WN PIV, overlaid with a semisolid tragacanth overlay, incubated at 37 C, and then fixed and stained with a MAB specific for WNV NS1 (provided by E. Konishi, Kobe, Japan), as described above.

Example 9

PIV Safety Studies

PIV safety was established by inoculation of different doses of virus (YFV 17D or WNV TX02 recovered from parental infectious cDNAs) or PIV into 3- to 4-day-old mice (outbred Swiss Webster, Harlan) by the intracranial (i.c.) route (20 ml volume), or 4-5 week old female mice (outbred Swiss Webster, Harlan) by the intraperitoneal (i.p.) route (100 ml volume). Mice were monitored for 14 days for signs of disease and death, animals that were moribund, and appeared to be unable to survive until the next day were humanely euthanized and scored as "dead" the following day.

Example 10

WN PIV Potency and Efficacy Studies

Selected animals inoculated with WN PIV as described above were euthanized and bled at 21 days post inoculation. Sera were harvested from the animals, pooled, and tested for their ability to reduce WNV focus formation on Vero cell monolayers using the methods described above. The remaining animals were inoculated with 1,000 inf.u (determined by focus-forming assay on Vero cells), corresponding to approximately 100 LD_{50} of the NY99 strain of WNV (Xiao et al., 2001), and animals were then observed for an additional 14 days as described above.

Example 11

Both YFV C- and YFV C-prM-E-Expressing Cassettes can Complement Replication of YFV PIV

The general strategy for complementation of a C deletion defect in the flavivirus genome is presented FIG. **1**. It is based on development of genomes lacking the C gene, and propagation of these pseudoinfectious viral genomes (PIV genomes) in cells expressing C (or all of the viral structural proteins), but not in normal cells. Replication in the latter cells, producing no viral structural proteins required for trans-complementation of the defect in PIV genome, leads to release of SVPs containing the critical protective immunogen E, but lacking the nucleocapsid containing C and the viral genetic material.

A recombinant YFV genome (YF PIV genome) was engineered to contain GFP in place of amino acid 26-93 of C, cloned in-frame with the rest of the polypeptide (FIG. **2**A). The expression of GFP provided a convenient way of determining the titers of genome-containing PIVs in the experiments. The deletion in the C-coding sequence from this PIV genome was expected to destroy the ability of C to form a functional nucleocapsid, but it was not expected to affect production of functional forms of prM and E. Thus, cells expressing this genome, which produced GFP fluorescence could not release infectious virus. However, infectious progeny was expected to be produced from "packaging" cells expressing high levels of C.

For rapid development of the cell lines for efficient PIV production, the Venezuelan equine encephalitis virus (VEEV) genome-based expression system (replicons) (Petrakova et al., 2005) was used. VEEV replicons are less cytopathic than replicons derived from other alphaviruses and readily establish persistent replication in some cell lines of vertebrate and insect origin. The expression cassettes were designed as double subgenomic constructs (FIG. **2**B), in which one of the subgenomic promoters was driving the expression of Pac, providing an efficient means to eliminate cells in the transfected cultures that do not contain the Pac-expressing VEEV replicon. The second subgenomic promoter was driving the transcription of subgenomic RNA encoding YFV structural proteins. To identify the most efficient packaging cassettes, VEEV replicons encoding either i)

YFV C with the signal peptide of prM, also known as anchored C (Lindenbach and Rice, 2001), (VEErep/C1/Pac), or ii) C with the signal peptide and 20 amino acid of prM (VEErep/C2/Pac), or iii) all of the YFV structural proteins (VEErep/C-prM-E/Pac). The rationale of the design was to retain the signal peptide in the C-coding cassettes that was expected to be essential for targeting C into proper cellular compartment.

The in vitro-synthesized VEEV replicon RNAs were transfected into BHK-21 cells and the Pur^R stable cell lines were selected over the next 4-5 days in the Pur-containing medium. During the first 2-3 days post transfection, replication of VEEV-derived RNAs caused growth-arrest, then, as described our previously (Petrakova et al., 2005), replication became less efficient and cells resumed their growth. The resulting Pur^R cultures were transfected with the in vitro-synthesized YF PIV RNAs, and at different times post transfection, titers of the released infectious particles, containing GFP-expressing genomes were determined (FIG. **2**C). Surprisingly, the presence of two different replicating RNAs (YFV- and VEEV-specific) in BHK-21 cells did not result in cytopathic effect (CPE), and maintained both resistance to Pur and expression of high level of GFP, indicating replication of both the VEEV replicon and YF PIV RNA. As shown in FIG. **2**C, cultures expressing both of these marker genes were capable of growing and required subpassaging (at ~1:5 ratio every 4 days) to prevent the cultures from reaching confluency. The experiments shown in FIG. **2** demonstrated that all three VEEV replicons were capable of supplying YFV C at levels sufficient for formation of infectious PIVs; no infectious particles were released from the naive BHK-21 cells transfected with the YF PIV RNA in the absence of VEEV replicons (data not shown). However, cells expressing these packaging cassettes differed in their ability to produce PIV. Constructs expressing YFV C followed by the prM signal peptide (anchored C; VEErep/C1/Pac) demonstrated the lowest level of YF PIV RNA packaging, compared to cassettes expressing longer protein sequences. The basis for the lower packaging efficiency is by the C1 construct is unclear, but this phenomenon might be explained by a requirement for a specific ordering of cleavage at the two nearby cleagage sties (NS2B/NS3 and signal peptidase) (Yamshchikov and Compans, 1994), and/or differences in distribution/stability of the C protein produced in these two different contexts. of the stability of this protein. Thus, after these experiments, VEErep/C1/Pac was eliminated from all further experiments. Both VEErep/C2/Pac and VEErep/C-prM-E/Pac replicons packaged YF PIV to the similar titers approaching above 10^7 inf.u/ml. Moreover, the release of PIV particles continued until the experiments were terminated, with each cell releasing ~20 infectious YF PIV per 24 h time period. The same cells were probably also releasing prM/E-containing SVPs lacking the nucleocapsid and genome, but this possibility was not further investigated.

Example 12

YF PIVs with Defective Genomes can be Produced at a Large Scale

The ultimate utility of PIV as vaccine candidates is dependent upon the ability to produce these particles at the scales needed, for instance, for commercial production. Reliance on an RNA-based trans-complementation system (VEEV replicons) for vaccine manufacture requires further standardization since there is a possibility of accumulation of mutations in the heterologous genes cloned into genomes of RNA viruses. The use of low-passage cell lines, is one of the solutions for overcoming this limitation. Alternatively, accumulation of mutations in the VEErep genomes can be minimized by repeated transfection of the replicon into naïve cells, or by production of packaged VEEV replicons followed by infection of naïve cells. The use of packaged VEE replicons was considered to be one simple and efficient means for establishing the packaging cell lines.

To efficiently produce PIVs, a technology that permits production of alpha virus replicon expressing cell cultures in previously packaged VEEV replicons was used. Briefly, VEEV replicons were packaged into VEEV infectious virions using previously described two-helper system (Volkova et al., 2006), into preparations that contained titers approaching 10^9 inf.u/ml. BHK-21 cells infected with these particles and selected in the presence of Pur could be used to obtain YFV structural protein-encoding cell cultures in 3-5 days. Following establishment, the VEErep/C2/Pac- and VEErep/C-prM-E/Pac-containing cell lines were infected with previously generated samples of YF PIVs at high (10 inf.u/cell) and low (0.1 inf.u/cell) MOIs. In all cases, the defective YFVs replicated productively (see FIG. **3**) and infected all of cells in the monolayers producing high titers of PIVs. Thus, rapid establishment of packaging cell lines by infecting cells with packaged VEEV replicons, followed by infection with PIVs appears to be a simple and efficient system a for large-scale production of PIVs with the deleted C sequence in the genome.

Example 13

Production of YF PIVs Using VEE Replicons Expressing Codon-Optimized Form of YFV C Gene

Another possible problem in using the packaging systems to support replication of defective viruses is recombination between the defective viral genomes and the RNAs encoding the trans-complementing gene(s). Such recombination might lead to generation of the infectious viruses. In the experiments described herein, infectious YFV using a plaque assay were never detected, but it was necessary to rule out the possibility that live virus can be formed in these cells.

In addition, the proteins encoded by many arthropod-borne viruses are expected to have evolved to utilize the translational machinery in two very different hosts. Thus, their codon usage is not expected to be optimal for expression in either host. Therefore, the C-coding sequence in the expression cassettes was modified to achieve two goals: i) to enhance the yield of C production and ii) to reduce possibility of homologous recombination between YF PIV genome and C-coding subgenomic RNA of VEE replicons. YFV C was synthesized using the codon frequency found in the most efficiently translated mammalian genes (FIG. **4**A). These silent mutations also disrupted the cyclization sequence required for flavivirus genome replication, thus, reducing the possibility of generating replication competent YFV in an event of recombination between YF PIV genome and YFV C-coding RNA of VEEV replicon.

The Copt gene was cloned into VEErep constructs, VEErep/Copt/Pac and VEErep/Copt-prM-E/Pac, using the same strategy as VEErep/C2/Pac and VEErep/C-prM-E/Pac, and trans-complementing Pur^R cell lines were established either by RNA transfection or by infecting the cells with packaged RNAs. Transfection of these cells with the in vitro-synthesized PIV genome RNA produced PIV with efficiencies that were similar to those selected with the cells expressing VEEV replicons expressing the non-optimized YFV C

gene (FIG. **4**B). However, the cells expressing the codon-optimized C proved to be a useful reagent in that they were capable of developing CPE and forming clearly visible plaques when infected with YF PIV and overlaid with agarose containing media with low concentration of FBS (FIG. **4**C). Thus, although codon optimization of YFV C gene did not alter PIV production from these cells, the cells expressing the codon-optimized YFV C represent a very useful system for evaluation of YF PIVs, particularly those expressing no fluorescent markers. In additional tests, a very good correlation was observed between the titers of the same samples determined in plaque-forming assays and GFP-foci assays.

Plaques formed by YF PIV were smaller than those of YFV indicating that structural proteins were most likely produced in cis finctio more efficiently in viral particle formation. The reason for attaining the ability to form plaques is not completely understood yet. However, it is speculated that YFV C has some level of cytotoxicity because of cell lines containing VEEV replicons expressing the codon-optimized version of this protein demonstrated lower growth rates (data not shown) than corresponding counterparts with replicon encoding natural C gene. Thus, YF PIV genome replication might lead to additional changes in the intracellular environment that were sufficient to cause CPE.

Example 14

PIVs can be Generated for Other Flaviviruses

To prove that PIVs can be easily generated for other flaviviruses, the strategy described above was applied to WNV. To this end, a WN PIV genome with a 35-amino acid-long C protein was created (FIG. **5**A). To package this WN PIV genome, a packaging cell line generated by transfection of BHK cells with a non-cytopathic VEEV replicon expressing WNV C/prM/E and Pac [BHK(VEErep/C*-E*-Pac) was used. To minimize the chance that recombination between WN PIV genomes replicating in this cell line and the VEErep RNA-encoded C protein could lead to generation of the infectious WNV, the WNV C-coding gene in the VEEV replicon was modified to contain clustered silent mutations in the WNV cyclization domain.

Media harvested from BHK (VEErep/C*-E*-Pac) cells transfected with the synthetic WN PIV genome were capable of producing antigen-positive foci in the packaging cells (FIG. **5**B) indicating that infectious WN PIV had been produced. However, only antigen-positive cells were detected upon infection of Vero cell monolayers with same samples (FIG. **5**C). Titers of up to 1×10^8 inf.u/ml of WN PIV were produced on the packaging cells, and as expected, WN PIV could be repeatedly passed on this cell line. Thus, using an established cell line, high titer stocks of WN PIV could be readily obtained using the same complementation system described above for YFV. Interestingly, in the case of the WNV packaging cell line and WN PIV, it was observed that the virus yields plateaued late in infection, simultaneously with the appearance of CPE (results not shown), whereas the cells co-replicating YF PIV genome and VEEV replicons continued to produce PIV for many days (FIG. **2**).

Example 15

Cells Infected with YF or WN PIVs Produce SVPs

To demonstrate that cells infected with PIVs produced SVPs, BHK-21 cells were either transfected with the in vitro-synthesized YF PIV RNA or infected with YF PIVs produced in C-expressing cells. The particles released from the BHK-21 cells were purified by ultracentrifugation, and analyzed by western blotting using a mouse monoclonal antibody (MAB) specific for E, D1-4G2 (Gentry et al., 1982). Both RNA-transfected and PIV-infected cells produced E protein that could be pelleted from the media (FIG. **6**A), indicating that it was present in a particulate form. Since these cells did not exhibit any CPE, and the samples were clarified at low-speed centrifugation prior to ultracentrifugation, it is unlikely that the E protein detected in the pelleted fraction represented cellular debris. Similarly, western blot analyses demonstrated that Vero cells infected with the WN PIV produced (before development of any signs of CPE) extracellular forms of E that were indistinguishable in size from those produced by WNV-infected Vero cells (FIG. **6**B).

To further evaluate the physical nature of the E protein released by PIV-infected cells, media collected from cells containing replicating PIV genomes only were subjected to sucrose density gradient analysis in agreement with published data (Schalich et al., 1996). SVPs were found in the fraction having 2% sucrose (FIG. **6**C). In the same experiment, YFV virions demonstrated high density and were detected in the fraction with 42% sucrose. E protein-containing particles that migrate at the expected size of WNV SVPs have also been detected in cultures infected with WNV PIVs. The presence of E in the media of PIV-infected cells was consistent with the production of SVPs by cells expressing only prM/E or TBEV RNA vaccines lacking a functional C gene.

Example 16

PIV Safety Potency, and Efficacy in Animals

Safety of WN and YF PIVs was established by i.c. inoculation of litters of 3 to 4-day-old mice. These studies showed that mice inoculated with WT YFV or WNV were quickly killed, and these viruses displayed a 50-percent lethal dose (LD_{50}) of approximately 1 PFU in these animals (Table 1). However, WN and YF PIVs inoculated into suckling mice at a dose of 2×10^6 inf.u failed to kill any mice (Table 1). Safety was further documented by i.p. inoculation of adult mice with wild type (wt) viruses and WN PIVs. These studies showed that the WN PIVs were completely safe in adult mice (Table 2). Furthermore, wt WNV killed a significant portion of adult mice, with an LD_{50} of less than 1 PFU, and doses of up to 3×10^6 inf.u of WN PIV failed to cause any death (Table 2). Most interestingly, however, is the finding that the WN PIVs were very potent immunogens (NEUT titers were detected with inoculation of as few as 30,000 inf.u), and 100% of the animals vaccinated with 3×10^4, 3×10^5, or 3×10^6 inf.u were protected from a $100LD_{50}$ challenge of the NY99 strain of WNV (Table 2).

TABLE 1

Safety of PIVs in suckling mice.

Inoculum[a]	Dose (inf.u)[b]	% Survival[c]	Average survival time[d]
WN PIV	2,000,000	100 (9/9)	NA[e]
WNV TX02	0.2	56 (5/9)	8.5 (+/−2.9)
WNV TX02	2	0 (0/9)	5.4 (+/−0.5)
WNV TX02	20	0 (0/8)	6 (+/−0)
WNV TX02	200	0 (0/10)	4.9 (+/−0.3)
YF PIV	2,000,000	100 (10/10)	NA[e]
YFV 17D	0.2	89 (8/9)	8 (+/−0)
YFV 17D	2	56 (5/9)	7 (+/−0)

TABLE 1-continued

Safety of PIVs in suckling mice.

Inoculum[a]	Dose (inf.u)[b]	% Survival[c]	Average survival time[d]
YFV 17D	20	11 (1/9)	6.9 (+/−2.4)
YFV 17D	200	0 (0/12)	6 (+/−0)

[a]Inoculated preparation, diluted in culture media with 10% FBS
[b]Delivered by i.c. route in a volume of 20 ml/animal
[c]Survival at 14 days postinoculation (live/dead)
[d]Average survival time from animals that died from infection (standard deviation)
[e]Not applicable

TABLE 2

Safety, potency and efficacy of PIV in adult mice

Inoculum[a]	Dose (inf.u)[b]	% Survival[c]	Average survival time[d]	NEUT titer[e]	% Protection[f]
none (diluent)	0	100 (8/8)	NA[g]	<1:40[h]	14 (1/7)
WN PIV	30,000	100 (10/10)	NA[g]	1:40	100 (8/8)
WN PIV	300,000	100 (10/10)	NA[g]	1:160	100 (8/8)
WN PIV	3,000,000	100 (10/10)	NA[g]	1:160	100 (8/8)
WNV TX02	1	40 (4/10)	8.5 (+/−1.4)		
WNV TX02	10	30 (3/10)	8 (+/−1.2)		
WNV TX02	100	10 (1/10)	7.8 (+/−1.4)		

[a]Inoculated preparation, diluted in culture media with 10% FBS.
[b]Delivered by i.p. route in a volume of 100 ml/animal.
[c]Survival at 14 days postinoculation (live/dead).
[d]Average survival time from animals that died from infection (standard deviation).
[e]NEUT titer of pooled sera collected from 2 animals at 21 days postinoculation (titer shown is the highest dilution giving 80% reduction of WNV foci formation).
[f]Protection from challenge with 100 LD_{50} of the NY99 strain of WNV demonstrated by survival at 14 days post-challenge; single survivor from the diluent-inoculated group showed signs of disease (hunched back, ruffled fur, and malaise) from days 8-14. None of the PIV inoculated animals displayed any signs of disease in the 14-day postchallenge observation period.
[g]Not applicable.
[h]NEUT liters in sera from unimmunized mice tested side-by-side with sera from the WN PIV-inoculated mice.

Example 17

Further Modifications to Increase the Yield and Safety of PIVs/RepliVAX

The present invention demonstrates that repeated passaging of RepliVAX did not result in recombination, but variants with enhanced growth were selected: The WNV RepliVAX has been repeatedly passaged on a cell line that encodes the WNV C protein. This C protein was produced by fusing a copy of the WNV C gene to a Pac gene driven by the subgenomic promoter of a non-cytopathic VEErep (Petrakova et al., 2005). In the resulting construct (VEErep/Pac-Ubi-C*), the ubiquitin (Ubi) gene was inserted in front of the C gene, and C was followed by a stop codon. In this context, a Pac-Ubi fusion protein would be produced along with a mature C protein (lacking the hydrophobic anchor; see FIG. **9**). The C gene in this VEErep (denoted as "C*") was further modified by insertion of 36 mutations that ablate the CS signal, converting this 11-base region from GUCAAUAUGCU (SEQ ID NO: 2) to GUgAAcAUGuU (SEQ ID NO: 3) while maintaining C coding capacity. This large number of mutations dramatically reduces the likelihood of homologous recombination, and furthermore, if recombination did occur between the genomes, the production of a replicationally active genome could not occur, since the resulting RNA would have unmatched CSs, preventing replication (FIG. **9**).

To test for the unlikely possibility of productive recombination, a clonal cell line was derived from BHK cells expressing VEErep/Pac-Ubi-C* {BHK(VEErep/Pac-Ubi-C*)}, and this cell line was used to passage the WN RepliVAX 10 times (in each case with infection at an MOI of 0.01), and the resulting RepliVAX was characterized in detail. To determine if this passage 10-(p10) population contained any live virus, Vero cell monolayers were infected at multiplicities of 0.1, 1, and 10 with the p10 WN RepliVAX, and washed extensively to remove extracellular RepliVAX. These monolayers were re-washed 24 hours later, and then harvested 2 days later. Passage of supernatant fluids from these cultures onto fresh Vero cell cultures failed to reveal any immunopositive cells when stained with a highly sensitive polyclonal antibody for WNV, indicating that RepliVAX had not productively recombined with the C protein encoded by the packaging cell line.

Interestingly, when the p10 WN RepliVAX was compared to p0 RepliVAX on the BHK(VEErep/Pac-Ubi-C*) cell line, the p10 RepliVAX produced polymorphic foci of infection, many of which were much larger than those produced by the p0 RepliVAX (FIG. **10**). Furthermore, p10 RepliVAX replicates 10 times higher than p0 RepliVAX at early time points, with an endpoint titer twice as high.

Analyses of the PCR products obtained from cDNA produced from Vero cells infected with this p10 RepliVAX demonstrated that there were no products that contained a full-length C coding region. However, sequence analyses of the C-prM junction of the product spanning these regions revealed that two mutations had arisen during passaging. As expected from the heterogeneous nature of the foci produced by the p10 RepliVAX on the packaging cells (FIG. **10**), both mutations were present as mixtures with the original RepliVAX sequence. One of the mutations, which appeared to be present over half of the nucleic acid population in these sequence reactions (sequenced in both directions), consisted of a AGC>uGC (S>C) mutation at the P4 position preceding the signal peptidase cleavage site (S(c)VGA|VTLS (SEQ ID NO: 4) in the RepliVAX genome. The second mutation, which was present in only about 30% of the amplified sequences (again in reactions completed in both directions) consisted of an AAG>AuG (K>M) at position P3 following the NS2B/NS3 cleavage site (QKKR|GGK(m)T) (SEQ ID NO: 5). Although these mutations are in the position of the deleted SL5, they do not alter predicted RNA structures. The rapid selection (only 10 growth cycles) of a better-growing RepliVAX is very exciting since it indicates that selection of better-growing variants is a powerful method to improve RepliVAX. The positions of these mutations was not unexpected since it is known that altering efficiency of NS2B/NS3 versus signal peptidase cleavage can influence flavivirus particle yield and infectivity (Keelapang et al., 2004; Lee et al., 2000; Lobigs and Lee, 2004; Yamschikov et al., 1997). Studies are continuing on selection of even better growing variants, and these two mutations are being targeted for insertion into second-generation RepliVAX constructs, to confirm their ability to work separately (or together) to improve RepliVAX yield and antigen production. Nevertheless, the data presented herein indicate that under these passage conditions: 1) no recombination occurred, 2) positive selection could be used to produce improved RepliVAXs.

Blind passage of JE RepliVAX similarly yielded better-growing variants with mutations in the same regions of the genome. The ability to blind passage RepliVAX products to produce better growing variants is a key feature of this invention, and a clear advantage over traditional LAV, where pro-

duction of better-growing variants is always complicated by the concern that these better-growing variants may have lost their attenuation in man.

Furthermore, the mutated, improved C-expression cassette (VEErep/Pac-Ubi-C*), which has been shown to be stable, and demonstrated freedom from recombination when used in a BHK cell line (not approved for human vaccine generation), has also been shown to be stable and useful for PIV propagation when introduced into Vero cells (an accepted cell line for the production of human vaccines). Specifically, RNAs corresponding to the VEE replicon have been introduced Vero cells from a certified seed using the same methods applied to BHK cells. Following introduction of the RNA into these Vero cells, the cells were maintained in serum free media (an important issue for vaccine generation) containing puromycin, and these cells were shown to be useful for PIV propagation. Under these propagation conditions, these cells have been shown to produce slightly lower titers of PIV than similarly derived BHK cells, but the VEErep/Pac-Ubi-C*-Vero cells hold up better under these culture conditions, permitting multiple harvests. FIG. **11** shows the production of PIV from these cells can be obtained for multiple harvests under serum-free conditions.

In summary, propagation of PIVs in cell lines that express C (especially C cassettes that contain the signal sequence of prM, or this region plus portions of the prM and E genes) can theoretically recombine with the PIV genome, producing a live virus that could cause disease, increasing the risk of the method of vaccine generation. To overcome this problem, the present invention demonstrated that cell lines for the propagation of WN PIV can be produced using a C protein that ends precisely at the NS2B/NS3 cleavage site, minimizing the chance of recombination at this region of the PIV genome, providing an advantage over other propagation methods in which cell lines encode RNAs that encode the portion of the anchor of C (that is also know as the signal peptide of prM) that are shared by the PIV.

To further enhance the safety of this C-expression cassette, the present invention demonstrated that the portion of the cassette that is used to make the VEErep-encoded C that complements the PIV genome (namely the first 30 codons encoding the amino acid sequence that are required to produce a replicating PIV genome due to underlying RNA elements required for viral replication) could be specifically mutated to produce a cassette that differs from the PIV genome at 36 nucleotide positions (introduced without altering the protein product) resulting in a C gene that has a dramatically reduced probability of recombination with the PIV genome (FIG. **9**). Furthermore, this mutated C gene was created to have three mutations in the cyclization signal (CS) that must be complementary to a CS in the 3'UTR of the PIV genome to allow viral replication, providing a further safety feature to prevent recombination (FIG. **9**). Finally, this C gene was inserted into the VEEreplicon following the selectable marker gene (pac), by using a ubiquitin gene to the intact C product from the resulting polyprotein (alternative self-cleaving sequences such as the auto-proteinase 2A of FMDV, or other related sequences could easily be substituted for ubiquitin). Creation of this single-polyprotein cassette provides the advantage of producing a genetically more stable VEEreplicon, reducing the chance of recombination within the propagating cell lines, eliminating the C-expression cassette, and reducing PIV yield. The resulting construct (VEErep/Pac-Ubi-C*, FIG. **9**) was introduced into BHK cells, and the cells were used to produce a clonal cell line expressing the VEE replicon using established methods (Fayzulin et al., Virology 2006).

One clonal cell line was examined after 18 passages from single-cell cloning, and found to have no evidence of any genetic deletion of the C cassettes (by RT-PCR), nor was it found to have any detectable mutations within the C-expression cassette. Most importantly, this cell line displayed similar ability to propagate the WNV PIV at a passage level as high as 41. Finally, following 10 passages of PIV on this cell line, no evidence of recombination producing PIV-recombinants capable of productive replication on cells that do not express the C cassette (namely WT Vero cells), and no evidence of introduction of C-encoding sequences into the PIV genome by RT PCR was observed.

Furthermore, to address concerns that PIV might recombine with flaviviruses in vaccines at the time of their vaccination, producing novel, virulent flaviviruses, the present invention demonstrated that WNV genomes with "unnatural" cyclization signals (CS) present in all known naturally circulating flaviviruses, can be generated that replicate to high levels. Evidence has been produced in several laboratories that the two CS found at the 5' and 3' ends of the genomes of all flaviviruses must be 100% complementary to provide productive viral replication (Khromykh et al. J. Virol., 2001; Lo et al., J. Virol., 2003; Alvarez et al., Virol., 2003). These studies also demonstrated that unnatural CSs could produce replicating genomes, as long as the CS were 100% complementary. However these investigators reported that all genomes with unnatural CS sequences had replication defects. By systematic analysis of CS in WNV genomes, specifically the testing the ability of carefully selected single base swaps to produce high-level replication, single-base changes, and subsequent double-base changes that permit high-levels of genome replication (FIG. **12**A) were identified. FIG. **12**B demonstrates that high-level replication of WNV genome with two-base substitutions is possible, and that genomes intentionally created with mismatched CS sequences (namely WT and the 2-base mutant) are not replicationally active. This mutation, and others like it, can therefore be utilized to produce PIV with a superior safety profile, since any recombinant virus resulting from a single-point genetic recombination between the CS-modified PIV vaccine and a virus circulating in areas where people are undergoing vaccination would not be replicationally active, and hence could not cause disease.

Example 18

BHK Cells Expressing WNV C Gene Maintain their Phenotype for Multiple Passages

Studies with a WNV C-expressing clonal cell line derived from BHK cells transfected with VEErep/Pac-Ubi-C* has demonstrated its long-term stability and utility in generating RepliVAX for several reasons. Firstly, these cells were useful for repeated passaging of RepliVAX. Secondly, side-by side focus-formation assays on cells at two different passage levels (passages 8 & 24 after single-cell cloning) showed indistinguishable WN RepliVAX titers and foci sizes. Finally, direct analysis of the sequence of the C-encoding cassette in these cells at the passage-24 level revealed no changes relative to the original VEErep sequence. Taken together these data indicate that cells harboring C-expressing VEEreps should be stable enough for use in the currently accepted master cell seed lot format used to produce human vaccines. Furthermore, the fact that VEEreps have already been used in human trials, make it likely that the application of the

VEErep-cell technology to Vero cells will not encounter any unexpected hurdles during regulatory approval.

Example 19

Lymphoid Tissue Targeting of WNV VLPs

As indicated supra, WNV VLPs are similar to RepliVAX, except in place of the flavivirus prM/E proteins, they can encode a reporter gene, or they can simply contain a flavivirus replicon without a reporter. VLPs can be readily produced in packaging cells expressing all three WNV structural proteins, and have been produced at high titer (Fayzulin et al., 2006). When 10^7 U of VLP were inoculated into mice, these animals produced 1,000 to 5,000 U/ml of type I interferon (IFN) in their serum 24 hr post-inoculation. IFN responses were produced by both ip and subcutaneous footpad injection (fp). Furthermore, popliteal lymph nodes dissected 24 hrs after fp inoculation with b-galactosidase-expressing VLPs contained large numbers of b-galactosidase-positive cells, indicating that VLPs, which enter cells in a manner indistinguishable from RepliVAX, are targeting important lymph organs. This result is consistent with the high levels of IFN elicited by VLP-injection and suggests that similar targeting is responsible for the high potency and efficacy of RepliVAX.

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Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. Further, these patents and publications are incorporated by reference herein to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

SEQUENCE LISTING

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Lys

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<220> FEATURE:
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Ala Val Asn Met Leu Lys Arg Gly Met Pro Arg Val Leu Ser Leu
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<210> SEQ ID NO 18
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<212> TYPE: RNA
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<223> OTHER INFORMATION: sequence of the West Nile RepliVax genome overlaps with sequence of VEErep

<400> SEQUENCE: 18

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What is claimed is:

1. A replication-deficient pseudoinfectious virus comprising:

a West Nile or Yellow Fever deletion mutant genome comprising a deletion within the nucleotide sequence encoding amino acids 26 to 100 of the capsid protein, wherein the deletion mutant genome cannot produce capsid-containing viral particles in a cell that does not express a capsid protein, wherein the deletion does not disrupt the maturation of the prM protein or the RNA sequence required for genome cyclization, and a complementary capsid protein.

2. The replication-deficient pseudoinfectious virus of claim **1**, wherein said virus is a chimeric virus comprising a heterologous prM-E cassette.

3. The replication-deficient pseudoinfectious virus of claim **2**, wherein the heterologous prM-E cassette is from a yellow fever virus, as West Nile virus, a dengue virus, a

tick-borne encephalitis virus, a Saint Louis encephalitis virus, a Japanese encephalitis virus, or a Murray Valley encephalitis virus.

4. The replication-deficient pseudoinfectious virus of claim **1**, wherein the deletion is of amino acids 26 to 93, 31-93, 31-100, or 26 to 100 of the capsid protein.

5. The replication-deficient pseudoinfectious virus of claim **1**, wherein said deletion mutant genome further encodes a marker protein or an antigen.

6. The replication-deficient pseudoinfectious virus of claim **5**, wherein the marker protein is a green fluorescent protein.

7. A cell culture system comprising:

a West Nile or Yellow Fever deletion mutant genome comprising a deletion within the nucleotide sequence encoding amino acids 26 to 100 of the capsid protein, wherein the deletion mutant genome cannot produce capsid-containing viral particles in a cell that does not express a capsid protein, and wherein the deletion does not disrupt the maturation of the prM protein or the RNA sequence required for genome cyclization; the deletion mutant genome being inside a cell that expresses a complementary capsid protein, wherein the cell does not express prM or envelope proteins.

8. The cell culture system of claim **7**, wherein the cell comprises a replicon encoding a codon-optimized version of the complementary capsid protein.

9. The cell culture system of claim **7**, wherein the cell comprises an alphavirus replicon encoding the complementary capsid protein.

10. The cell culture system of claim **9**, wherein the alphavirus is Venezuelan Equine Encephalitis Virus.

11. A method of producing a replication-deficient pseudoinfectious virus comprising:

providing a West Nile or Yellow Fever deletion mutant genome comprising a deletion within the nucleotide sequence encoding amino acids 26 to 100 of the capsid protein, wherein the deletion mutant genome cannot produce capsid-containing viral particles in a cell that does not express a capsid protein, wherein the deletion does not disrupt the maturation of the prM protein or the RNA sequence required for genome cyclization;

providing a cell that expresses a complementary capsid protein;

inserting the deletion mutant genome into the cell; and

culturing the cell to produce a replication-deficient pseudoinfectious virus.

12. The method of claim **11**, wherein the deletion mutant genome comprises a heterologous prM-E cassette.

13. The method of claim **12**, wherein the heterologous prM-E cassette is from a yellow fever virus, a West Nile virus, a dengue virus, a tick-borne encephalitis virus, a Saint Louis encephalitis virus, a Japanese encephalitis virus, or a Murray Valley encephalitis virus.

14. The method of claim **11**, wherein the cell comprises a genetically engineered replicon derived from a viral vector.

15. The method of claim **14**, wherein the replicon encodes a codon-optimized version of the complementary capsid protein.

16. The method of claim **14**, wherein the replicon comprises the unnatural cyclization sequence of SEQ ID NO:3.

17. The method of claim **11**, wherein the deletion mutant genome comprises one or both of altered C-prM junction sequences SEQ ID NO:4 and SEQ ID NO:5.

18. The method of claim **11**, wherein inserting the deletion mutant genome into the cell comprises transfecting with in vitro synthesized replicon RNAs, transfecting with plasmid DNAs designed to synthesize functional alphaviral replicons from cellular RNA-polymerase II-specific promoter, or by infecting with alphaviral replicons packaged inside alphaviral structural proteins.

19. The method of claim **14**, wherein the replicon is an alphavirus replicon.

20. The method of claim **19**, wherein the alphavirus is Venezuelan Equine Encephalitis Virus, Sindbis virus, Eastern Equine Encephalitis virus, Western Equine Encephalitis virus, or Ross River virus.

21. A pharmaceutical composition, comprising the replication-deficient pseudoinfectious virus of claim **1**.

22. The replication-deficient pseudoinfetious virus of claim **1**, wherein the deletion mutant genome comprises one or both of altered C-prM junction sequences SEQ ID NO:4 and SEQ ID NO:5.

23. The method of claim **11**, wherein the cell does not express prM or envelope proteins.

24. The cell culture system of claim **8**, wherein the replicon comprises the unnatural cyclization sequence of SEQ ID NO3.

25. The cell culture system of claim **7**, wherein the cell further expresses a marker gene.

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